Analysis of Mold and Yeast Phosphoproteomes in the Dimorphic Fungus *Penicillium marneffei*

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ABSTRACT

Penicillium marneffei is a dimorphic fungus capable of multinucleate mold growth at 25°C and unicellular yeast growth at 37°C. Mostly prevalent in Southeast Asia, *P. marneffei* is pathogenic in immunocompromised individuals as the cause of penicilliosis. The switch from mold to yeast is a prerequisite for pathogenicity and the molecular mechanism responsible for such change is under investigation.

As the organism changes in cell morphology, molecular modifications are made in the processes that affect both cell growth and cell differentiation. Both of these programs rely greatly on the post translational modification of proteins known as protein phosphorylation. A combination of the activity of both protein kinases and phosphatases produces entities known as signaling pathways which enable the cell to coordinate cellular events and respond to external signals. The current phosphorylated state of all proteins in a cell at a given time point is known as the phosphoproteome. This study is the first to produce a phosphoproteomic analysis of mold and yeast cells in *P. marneffei.*

To study phosphoproteins in *P. marneffei,* whole cell proteins were extracted from mold and yeast cells. Proteins were then quantified using the Bradford assay and were separated by size using one dimensional gel electrophoresis. Gels were stained using Pro-Q Diamond Phosphoprotein Gel Stain for detection of phosphoproteins and comparative analysis was performed using Quantity One. Cells were also treated with rapamycin to determine morphological and phosphoproteomic changes brought about by the inhibition of the protein kinase TOR.

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INTRODUCTION

Dimorphism

There are two distinct morphologies common to fungal growth and development. One such type is growth as a mold, a multicellular, filamentous form comprised of structures known as hyphae. The other morphology is the unicellular type known as yeast. Some fungi possess the ability to grow as either a mold or yeast depending on the condition of their external environment. This attribute is known as dimorphism. Dimorphic fungi may grow as pseudohyphae in which their cells undergo cytokinesis but do not divide. This produces chains of elongated cells. True hyphae may also be produced in which elongated tubes are compartmentalized by structures known as septae which function to separate nuclei within the hyphae. In contrast to the filamentous growth of psuedohyphae and true hyphae, dimorphic fungi are also capable of growth like that of yeast. In this growth form, mitotic divisions by either budding or cellular fission produce the single cell morphology of yeast cells. Unlike the multinucleate hyphal form, yeast cells are uninucleate (20).

There are many factors that can trigger dimorphic fungi to undergo a morphological change. Factors such as nutrient availability, pH, and temperature are known to play roles in this process. Such as in the case of *Candida albicans,* the transition from yeast to a hyphal cell form can be brought about if the fungi experiences a neutral pH, or increased temperatures. Certain compounds such as CO_2, N-acetylglucosamine, and amino acids can also influence morphogenesis in *C. albicans* (2). Temperature induced dimorphism is of particular interest due to its role in the ability of pathogenic dimorphic fungi to cause systemic disease.

Dimorphism and disease

Systemic dimorphic fungi are a group of fungi that cause systemic disease in humans. There are six pathogens that can be categorized as such: *Blastomyces dermatidis, Coccidioides immitis, Histoplasma capsulatum, Paracoccidioides brasiliensis, Sporothrix schenkii, and Penicillium marneffei.* All of the above pathogens are fungi that undergo temperature induced phase transition from mold to yeast. Typically, the mold form is prevalent at 25°C whereas growth in the yeast form occurs at 37°C (14). Studies have shown this conversion does not function solely as a survival adaptation to increased temperatures found in mammals and birds; however it is a necessary precursor for pathogenesis. This is best exemplified in an over 20 year old study done by Medoff et al where it was found that mold cells of *H. capsulatum* treated with *p*-chloromercuriphenylsulfonic acid, or PCMS, become locked in the mold phase and thus are unable to convert to yeast when incubated at 37°C. Instead, they continue to grow as mycelia at this increased temperature. When PCMS treated mold cells are subsequently transferred to mice, they are unable to cause disease (15).

The role of dimorphism in virulence was also highlighted in studies involving the dimorphic fungus *Candida albicans,* which forms invasive mycelia in human hosts. When examining the antifungal activity of lycopene on *C. albicans*, it was found that yeast cells cultured with the botanical compound do not form the invasive mycelia cell type and instead remain as yeasts even when exposed to fetal bovine serum, a typical dimorphism inducing agent in the fungus. Thus the mechanism of antifungal activity lycopene is in part due to its ability to inhibit the dimorphic change in *C. ablicans* (21).

More recently, work was done involving the human pathogen *Cryptococcus neoformans.* This fungus exhibits morphological changes *in vivo* with the formation of larger yeast cells

known as “titan” cells, which can be found in the lung tissue of infected hosts. A 2009 study by Okagaki et al tested titan cells for their physical and biochemical interactions with host immune cells. It was found that host macrophages were unable to phagocytose titan cells as they are too large for engulfment. To examine the effects of chemical stresses, namely nitosative and oxidative, often implied by host cells, titan cells were separately treated with sodium nitrate ($NaNO_3$) and tert-butyl hydroperoxide (TBHP). It was found that titan cells were resistant to both extreme types of chemical environments as compared to normal cells, which exhibited slowed growth or death when treated as a control with $NaNO_3$ and TBHP respectively (19).

These findings show the importance of dimorphism in disease causing fungi, where dimorphic changes are a crucial step in the mechanism of pathogenicity. Such as the case of the dimorphic fungus *Penicillium marneffei,* in which phase transition from mold to yeast is required for virulence (8).

***Penicillium marneffei*- dimorphic fungal pathogen**

Penicillium marneffei is a dimorphic fungus that displays temperature dependent phase transition from mold at 25°C to yeast at 37°C and it is the causative agent of penicilliosis, a disease which affects immunocompromised individuals, namely, HIV patients. This disease is most common in Southeast Asia, especially in areas where HIV infection is widespread. Since the late 1980’s, a direct relationship has been established between the number of penicilliosis cases and the number of HIV affected individuals in Thailand, with a number of cases in immunocompromised patients who traveled through the area (24). *P. marneffei* is also known to infect bamboo rats, where it was first discovered in 1956. It is believed that the rats are not the main reservoir of penicilliosis infection as it is unclear what the environmental source may be;

although it is known that the rainy season delivers an increase of infection in endemic regions (24, 8).

Penicilliosis symptoms and treatment

Symptoms common to penicilliosis include fever, overall discomfort, weight loss, anemia, cough, and skin lesions (26, 24, 10). Further investigation of infected patients at Queen Elizabeth Hospital in Hong Kong from 1994 to 2004 revealed lymphadenopathy and hepatomegaly. Splenomegaly was also noted in 15% of patients. Many symptoms found in immunocompromised patients can often be attributed to other sources of infection associated with a debilitated immune system. Skin lesions, however, are noted often in cases of penicilliosis, and are a significant factor in diagnoses.

Successful treatment relies on prompt diagnosis as penicilliosis is often fatal when undetected. Once a diagnosis has been made, treatment begins with use of IV amphotericin B which is then followed up with oral administration of the antifungal drug intraconazole. Continued use of intraconazole is important to prevent relapse of penicilliosis (24,10).

Life Cycle and Development in *Penicillium marneffei*

There are three main stages of cellular development in *P. marneffei.* At 25°C the fungus can grow either as a filamentous mold containing multiple nuclei per cell or as a conidium, the asexual reproductive spore. The third growth form, also the pathogenic form, occurs at 37°C as a unicellular yeast. It is important to note the correlation of human body temperature of 37°C and the development of yeast at this same temperature (1, 24).

Growth as a filamentous mold begins with germination of conidia at 25°C and subsequent formation of a germ tube which is the result of polarized growth in the conidium. The germ tube then elongates and branches as septae divide the tube into multinucleate cells. This

type of growth, along with its characteristic asynchronous pairing of nuclear and cellular division, produces hyphae.

Upon the establishment of hyphal growth at 25°C asexual development may take place. This type of maturity usually requires that the fungus be in contact with air in order to proceed. Asexual development involves the formation of specialized uninucleate cells known as metulae and phialides which together make up structures called conidiophores. Conidia are generated from the phialides in a chain-like fashion by which the newest conidia push more mature ones farther from their origin. This mode of development is termed basipetal.

Development as pathogenic yeast occurs at 37°C in *P. marneffei* and begins similar to that of hyphal growth as the conidia germinates to produce hyphal structures. These structures formed at 37°C differ from those at 25°C as they are shorter, highly branched, uninucleate hyphae separated by a double septae. Eventually, cellular material between the septae dissipates as the segments separate in a process known as arthroconidiation. The result of this process is single elongated cells called arthroconidia. Arthroconidia contain a single nucleus and divide by fission after nuclear division occurs. Growth and division in this manner produce the yeast stage of *P. marneffei*.

Developmental Regulation in *P. marneffei*

Recently, studies have been conducted to uncover how cellular events such as conidiation, germination, and morphogenesis in *P. marneffei* are regulated at the molecular level. In 2000, a study by Borneman et al examined the role of the transcriptional regulator *abaA* in *P. marneffei. AbaA* is known to be a player in cellular development in many organisms including conidiation in *Aspergillus nidulans* and pseudohyphal growth in *S. cerevisiae* (3). Gene expression studies in *P. marneffei* shows that *abaA* RNA transcripts are most abundant during

conidiation and the yeast phase. The involvement of *abaA* in conidiation and yeast morphogenesis was further exemplified by the failure of deletion strains to properly produce phialides at 25°C and uninucleate yeast cells at 37°C.

Another transcriptional regulator, RfxA, has recently been examined in *P. marneffei* (6). RfxA is a helix turn helix DNA binding protein with potential targets such as regulatory cell cycle genes including those involved with mitotic-spindle dynamics and exit from mitosis. RNA interference was used to silence *rfxA* in *P. marneffei* and resulted in varying effects at both 25°C and 37°C. RfxA was shown to regulate growth of hyphae and development of conidiophores at 25°C as RNAi strains were defective in producing effective forms of either growth mode. At 37°C, RNAi strains failed to properly produce septae during arthroconidiation and thus were unable to form yeast cells indicating RfxA's regulatory role in morphogenesis.

The role of biological signaling pathways in dimorphic fungi such as *S. cerevisiae* and *C. albicans* has raised interest in similar pathways in *P. marneffei* (1). Pseudohyphal growth in *S. cerevisiae* is known to occur when the G-protein, Gpa2, activates a cAMP-PKA pathway (27). G-proteins have also been examined in *P. marneffei,* namely the G-protein GasA, which was also found to activate a cAMP-PKA pathway. Mutants produced to have a constitutively active form of GasA were incapable of producing conidia, indicating a negative role in asexual development. This result is further confirmed by the inactivation of GasA signaling which was shown to cause production of conidia in conditions unfavorable to asexual development in wild type strains. Although GasA seems to be highly involved in the process of conidiation, mutant strains showed normal yeast phenotypes indicating it plays little or no role in yeast morphogenesis.

Downstream of G-proteins are kinases that activate cascades such as cAMP-PKA and MAPK, which are important in growth regulation. In *S. cerevisiae,* the p21-activated kinase,

Ste20p, activates a MAPK cascade during the initiation of psuedohyphal growth (4). The p21-activated kinases of *P. marneffei* PakA and PakB have been studied for their roles in various developmental programs (4, 5). It was found that PakA is essential in conidial germination at 37°C, as deletion strains exhibited defects or failed to germinate altogether after four days of incubation. The involvement of PakA in germination is thought to be due to its role in establishment of polarized growth of the conidia as well as the developing yeast cells, arthroconidia. Analysis of PakA using a fusion protein show that the kinase is found in concentrated amounts in areas where polarized growth is occurring, such as the apex of hyphae during arthroconidiation at 37°C (4). The second p21-activated kinase found in *P. marneffei,* PakB, has been shown to play a role in *in vivo* development of yeast cells (5). PakB is unique because it appears to respond to signals from host macrophages instead of responding to temperature as there is no clear role for *in vitro* yeast cell development at 37°C. Phagocytosis via macrophages leads to an increase in the production of PakB. Data also suggests that PakB is involved in polarized growth of hyphae at 25°C as well as production of conidia. There is no clear role of PakB in germination at 25°C making the process unique from germination at 37°C, which requires PakA.

Aside from p21-activated kinases, another type, histidine kinases, have been shown to regulate various processes in pathogenic fungi via two-component signaling pathways (25). A 2006 study by Nemecek et al concluded that the hybrid histidine kinase, *DRK1,* regulates dimorphism and virulence in both *B. dermatidis* and *H. capsulatum* (17). Two- component signaling pathways involve a cell's ability to respond to its external environment via stimulation of autophosphorylation of histidine residues on histidine kinase receptor proteins which in turn phosphorylate response regulator proteins that relay the signal to various regions of the cell.

Through genomic analysis, researchers were able to identify a histidine kinase gene in *P. marneffei,* known as *PmHHK1.* Using double stranded RNA interference, the function of this gene in *P. marneffei* was examined. It was found that *PmHHK1* is involved in the regulation of morphogenesis as dsRNAi strains showed incorrect morphologies. The rate of growth and production of conidia was also adversely affected by silencing of *PmHHK1.* The importance of histidine kinases and two-component signaling in other pathogenic fungi makes the study of phosphorylation in *P. marneffei* necessary as these molecules as well as their downstream counterparts may be potential candidates for targets of novel drug treatments (25).

Protein Phosphorylation

Aside from regulating proteins by transcriptional regulation, cells can also modify proteins post-transcriptionally, resulting in alteration of their functional state. These types of alterations are called post-translational modifications, or PTM. Science has so far revealed over 200 different types of PTM, one of which stands out as the most common and most studied: protein phosphorylation. Playing a role in varieties of cellular processes such as cell cycle control, signal transduction, transcriptional and translational regulation, proliferation, differentiation, homestasis, and metabolism, protein phosphorylation involves the covalent addition of a phosphate group to an amino acid side chain of a given protein. This process is often considered to be as a molecular switch, turning a particular protein or pathway "on" or "off." Specialized proteins known as kinases are responsible for the addition of phosphate groups to target proteins, most often to serine, threonine, or tyrosine residues in eukaryotes. This can affect the protein in many ways, such as changing its shape. There are often many regions within a given protein that are subject to phosphorylation by one or many different kinases. This allows for proteins to take on several different functions without changes in the amino acid sequence. A

key concept leading to the effectiveness of protein phosphorylation as a PTM is that it is entirely reversible, giving cells further immediate control of protein activity. Acting as a counterpart to protein kinases, specialized proteins known as phosphatases are responsible for removing phosphate groups from proteins. This process is known as dephosphorylation. The collective action of kinases and phosphatases work to regulate the overall activity of proteins and pathways within the cell at any given time (9, 23).

Cell growth and proliferation are two important processes governed by the action of protein phosphorylation. It is crucial that they operate in a coordinated way with one another, as to maintain consistent cell size. The protein kinase TOR, or target of rapamycin, works to manage these processes and keep them in synch (11). TOR is a serine/threonine kinase composed of 2549 amino acids resulting in an approximate molecular weight of 304,000 Da. It is able to manage the cell's growth and progress through the cell cycle by responding to external components such as growth factors and available nutrients. Its name is derived from its sensitivity to the bacterial toxin rapamycin, which acts to inhibit the kinase. This type of action leads to a decrease in cell growth, the rate of cell cycle progression, and cellular proliferation. Regulation of protein biosynthesis is a primary mechanism of TOR's function as its main downstream targets, ribosomal protein S6 kinases (S6Ks), and eukaryotic initiation factor 4E (eIF4E), are both involved in protein translation. These targets, as well as TOR itself, have emerged as potential novel drug targets in treatment of tumors.

Another set of kinases that are especially important in cell division and regulation are the cyclin- dependent kinases, or cdks, which are highly conserved proteins which function to regulate the cell cycle (18). As their name implies, cdks are regulated primarily by proteins called cyclins. Cylcins are known to appear in varying concentrations throughout the cell cycle,

depending on which stage of the cycle they influence. The kinase activity of cdks, while dependent on the binding of cyclin subunits, is also subject to regulation by phosphorylation. This phosphorylation event allows for full activation of kinase activity as Cdk2 activity is known to increase 80 to 300 times in its phosphorylated state. This increase is in addition to the conformational changes brought about by cyclin binding. Certain cdks, such as the Cdc2-cyclin B and Cdc2-cyclin A complexes, require phosphorylation for full activation. In the case of Cdc2-cyclin B, the binding of cyclin B has a minimal effect on its kinase substrate, and binding of cyclin B to Cdc2 is only possible once phosphorylation has occurred (16).

Phosphoproteomes

The complete set of phosphorylated proteins in a cell is known as the cells phosphoproteome (12). The study of such an entity is important in biology as it allows for further understanding of the whole system of signaling pathways and other biological processes regulated by phosphorylation (13). Currently, there are several methods used to study phosphoproteomes, most of which can be broken into three phases: separation of whole cell protein extract, tagging of phosphoproteins, and identification of phosphoproteins. Figure 1.1 is a flowchart depicting processes involved in various methods for study of phosphoproteomes (9). Highlighted in this figure is a method concerning the use of one dimensional gel electrophoresis (1DGE) and Pro-Q Diamond Phosphoprotein Gel stain. Many approaches used in studying phosphoproteins include the use of radioactive phosphate [^{32}P] during incubation of cells.

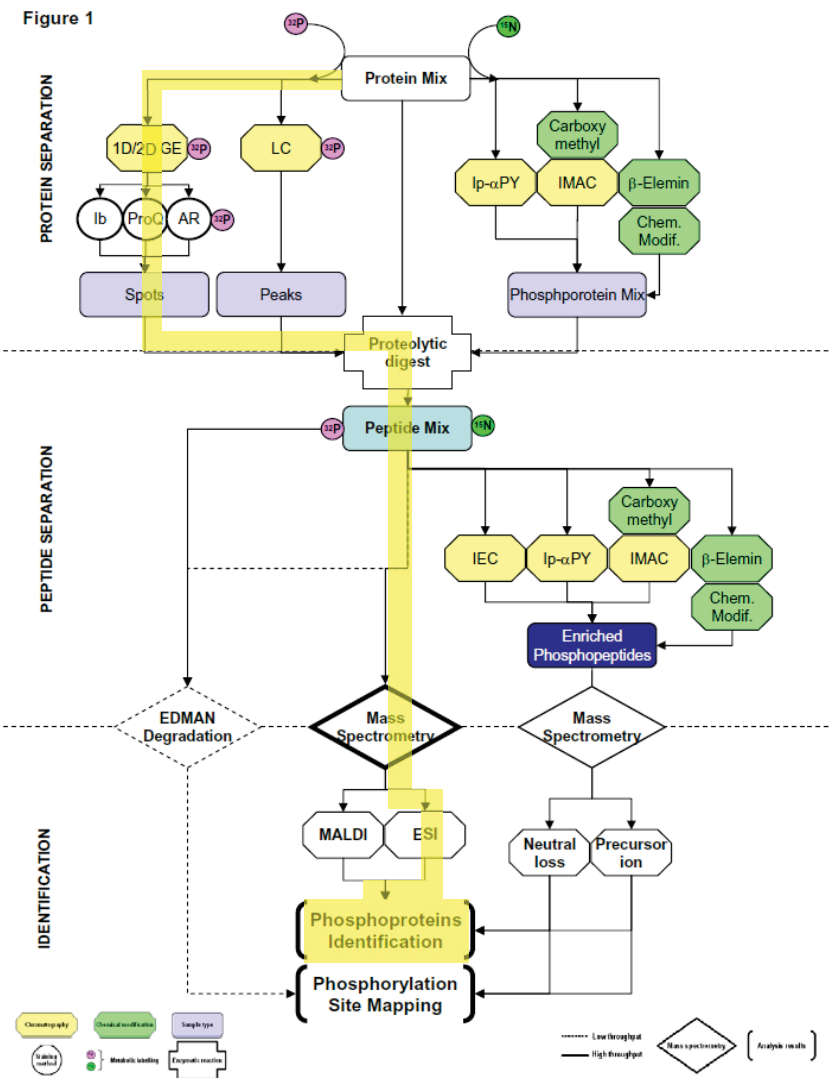


Figure 1.1. Flowchart of phosphoproteomic methods. This figure depicts several methods for the isolation of phosphoproteomes. The use of 1DGE and Pro-Q Diamond staining for phosphoproteome study has been highlighted.

Although a useful technique, limitations exist in the detection of some proteins in which a slow turnover of phosphate exists. In these cases, only a small amount of [^{32}P] is incorporated into the cell therefore leaving some proteins of interest unlabeled. Another technique has emerged which uses antibodies against phosphoproteins. In this case, proteins separated by 2DGE are blotted onto a membrane and are exposed to antibodies against the three most commonly phosphorylated residues: serine, threonine, and tyrosine. Using this method, small amounts of phosphoproteins,

including concentrations in the fmol range, may be detected. Limitations such as steric hinderance of antibody recognition sites, and with competence of antibodies to detect certain epitopes are present with this technique, however its usefulness grows as better antibodies are designed and produced (13).

SPECIFIC AIMS

Considering the importance of the involvement of protein phosphorylation in specific cellular events such as growth and differentiation, and the significance of dimorphic changes required for onset of disease by pathogenic fungi, this proposed study will analyze the phosphoproteomes of both the mold and yeast cell types in the dimorphic fungus *Penicillium marneffei*. The specific aims of this study are:

1. To determine the changes in phosphoproteomes that correlate with morphological changes from mold to yeast during the process of phase transition in *Penicillium marneffei.*

2. To determine the downstream substrates of TOR in *Penicillium marneffei* by analysis of phosphoproteomic changes caused by the drug rapamycin.

This study hypothesizes that phosphoproteomic profiles generated will show differences among phosphorylation patterns in mold and yeast cells as a result of differential kinase and signal transduction activity crucial to the process of differentiation during the dimorphic change. Differential profiles of the *P. marneffei* proteome have been shown by Chandler et al in 2008, however, a study of the phosphoproteome has yet to be done (7). It is also hypothesized that proteins of interest will be specifically involved in the process of cellular differentiation and other changes which occur during the dimorphic switch, and will be identified as such. In regards to the determination of TOR substrates, it is hypothesized that growth in the presence of the drug

rapamycin will produce changes in the phosphoproteome thus leading to the identification of target proteins.

METHODS

Harvesting of Conidia and Growth of Cells

The *Penicillium marneffei* F4 strain was used for all experiments and was maintained on potato dextrose agar (PDA). Upon ten days of growth at 25°C in 150 cc culture flasks, cells were removed from the agar using a cell scraper and sterile water. The cell and water solution was then filtered using sterile glass wool filters attached to 50 mL conical tubes centrifuged at 1000 RPM for 30 seconds. This was done to separate conidia from hyphae and resulted in a stock solution of conidia from which a dilution was made for cell counting. A hemocytometer was used to count conidia and thus determine the concentration of the stock solution. From the stock solution, mold and yeast cultures were prepared by inoculating 50 mL of sabouraud dextrose broth (SAB) per culture with a concentration of 1×10^7 conidia per mL in 250 mL sterile Erlenmeyer flasks. The cultures were then grown for 24 hours in orbital water baths at 25°C for mold cultures, and 37°C for yeast cultures. For cultures treated with rapamycin, 10 ug of rapamycin was added to each 50 mL culture for a final concentration of 200 ng/mL 12 hours after broth inoculation. At exactly 24 hours, cultures were rescued from water baths and placed on ice to stop any further growth. Microscope slides were made to examine if proper formation of mold and yeast cells had occurred as well as to check for contamination. On occasion, photomicrographs were taken using SPOT image capture software and camera. Cells were then separated from broth by centrifugation at 15,000 RPM for 20 minutes at 4°C. Upon separation, cells were twice washed with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) by centrifugation at 15,000 RPM for 15 minutes at 4°C. Excess TE buffer was removed and cells were pelleted for storage at -80°C.

Protein Isolation and Quantification

Cell pellets were retrieved from the -80°C freezer and were maintained on ice throughout the entire homogenization process. Acid washed glass beads were added in an equal volume to the cell pellet as well as 800 μL of lysis buffer (20 mM Tris-HCl, pH 7.6, 10 mM NaCl, 0.5 mM deoxycholate, 40 μL/mL protease inhibitor cocktail) and 20 μL of phosphatase inhibitor buffer (250 mM NaF, 50 mM β-glycerophosphate, 5 mM sodium orthovanadate). Phosphatase inhibitor buffer was prepared fresh before each use. The samples were then homogenized using the Mini Beadbeater™ from Biospec Products in 30 second intervals for a total of 4 minutes. Samples were placed on ice in between bead beating sessions. After the homogenization process, samples were centrifuged at 6,000 g for 10 minutes at 4°C. The supernatant was then transferred to preweighed 1.5 mL microcentrifuge tubes and protein was precipitated from solution using Trichloroacetic acid (TCA) at a volume 20% that of the supernatant. TCA precipitation occurred after 20 minutes of ice incubation and protein pellets were then three times washed with acetone (500 μL of cold acetone added, vortexed for 1 minute, then centrifuged for 1 minute at 3,000 RPM). Protein pellets were allowed to dry in a sterile biological safety hood with ventilation fan for 1 to 2 hours and were then resuspended in modified sample buffer (MSB) (2 M thiourea, 7 M urea, 4% w/v 3-[(3-cholamidopropyl)dimethylamino]propanesulfonic acid, CHAPS, 1% w/v dithiothreitol, DTT). For 5-15 mg protein pellets, 500 μL of MSB was used, and for 16-30 mg pellets, 750 μL of MSB were used. Once resuspended, samples were stored at -80°C.

Protein samples were quantified using a modified version of the Bradford Assay. A standard curve was constructed using known concentrations of bovine serum albumin (BSA) ranging from 10 to 40 μg/μL. For unknown samples, 10 μL of sample was added to each tube.

The remaining contents of each tube consisted of 80 μL of sterile H_2O, 20 μL 0.1 M HCl, 10 μL 2DE Buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 25 mM spermine base, 50 mM DTT) and 4 mL of Bradford dye (100 mg Coomasie Brilliant Blue G-250 dissolved in 50 mL 95% ethanol, 100 mL 85% (w/v) phosphoric acid added and solution diluted to 1 L with water). Using the BioRad Smart Spec™ Plus spectrophotometer, absorbancies at 595 nm were recorded and a standard line equation was generated in Microsoft Excel. The absorbancies of unknown samples at 595 nm was then used to determine the unknown concentrations.

One-Dimensional Gel Electrophoresis

Whole cell protein samples were separated by size using one-dimensional gel electrophoresis. All gels were run using 16 cm gel plates, 1 mm spacers and the BioRad Protean II xi cell. For each run two gels were cast with the resolving gel typically prepared on the previous day. All resolving gels consisted of a 10% polyacrylamide solution mixed in 65 mL quantities for two gels (32.5 mL ultrapure H_2O, 16.25 mL 40% acrylamide, 16.25 mL resolving gel buffer, 65 μL tetramethylethylenediamine, TEMED, 216.6 μL 10% ammonium persulfate, APS). Resolving gels were then stored at 20°C until day of use and were not stored longer than 5 days. On the day of the gel run, a 5% stacking gel was prepared in 15 mL quantities for two gels (9.4 mL ultrapure H_2O, 1.9 mL 40% acrylamide, 3.75 mL stacking gel buffer, 19.2 μL TEMED, 52.5 μL APS). The stacking gel was poured over the resolving gel and a 25 well Protean II xi comb was used to create sample wells. Samples were prepared for electrophoresis by mixing with 25% v/v 4X SDS Protein Sample Buffer (40% glycerol, 240 mM Tris/HCl pH 6.8, 8% sodium dodecyl sulfate, SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol). Originally, a gradient of protein amounts was loaded into sample wells ranging from 10-30 μg until it was

determined that wells containing 20 μg and 25 μg of protein yielded optimal imaging results. From then on samples were loaded in 20 μg and 25 μg quantities. Each gel also contained one standard lane with 10 μL of Mid/Low Range Protein Molecular Weight Marker from Amresco. Once samples and standard were loaded into wells, gels were run in pairs at a constant 40 mA per gels using the BioRad Power Pac 3000. The central cooling core of the Protean II xi cell was attached to a refrigerated water bath to ensure that the gels did not over heat during each run. Total run time was approximately 2.5 hours per run.

Pro-Q Diamond Phosphoprotein Gel Staining and Imaging

Upon completion of electrophoresis, resolving gels were separated from stacking gels and were stained using Pro-Q Diamond Phosphoprotein Gel Stain from Invitrogen™ to reveal only phosphoproteins in the separated protein sample. The staining protocol was derived entirely from the manufacturers manual, with the only variations including preparing in- house fixing solution and destain. Gels were first fixed for 1 hour with gentle agitation in fixing solution (40% methanol, 10% acetic acid). The fixing step was followed by three wash steps each using a large volume of ultra pure water with gentle agitation for 10 minutes. Every measure was taken to ensure that the stain and destain steps were performed in the dark as Pro-Q is extremely light sensitive. For the stain step, plastic containers (previously spray painted black to shield from light) were designated only for Pro-Q staining. To each gel container, 320 mL of Pro-Q (10X volume of gel) was added and allowed to stain for 90 minutes on gentle agitation in complete darkness. Upon completion of staining step, gels were destained three times in 500 mL of Pro-Q Destain Solution (20% acetonitrile, 50 mM sodium acetate, pH 4.0) for 30 minutes in complete

darkness. Gels were then twice washed with a large volume of ultra pure water for 5 minutes before imaging with the Pharos FX™ Plus Molecular Imager from Bio Rad.

SYPRO Total Protein Gel Staining and Imaging

After gels were stained and imaged for phosphoproteins, SYPRO® Ruby protein gel stain (Bio Rad) was used to stain for total protein. Gels were placed in SYPRO on gentle agitation and allowed to stain overnight. The following day, gels were twice washed in a large volume of ultra pure water for 5 minutes each prior to imaging with Pharos FX™ Plus Molecular Imager from Bio Rad. All gel results were produced in triplicate.

Gel Analysis

Computer analysis of both Pro-Q Diamond and SYPRO stained gels was performed using Quantity One (Bio Rad) to produce graphical and numerical comparisons of gel lanes as well as lane reports containing molecular weight data. Once a gel image was obtained, lanes were selected for analysis using the "create lane" tool. Individual bands could then be detected by the software using the "detect bands" command. Using the "compare lanes" feature, selected lanes were plotted graphically with each band represented as a data point on an X- andY- axis. In this case, Rf, or relative mobility of a band is plotted on the X axis and pixel intensity is plotted on the Y axis. From this data, particular bands were then selected for excision.

Protein Band Excision

Protein bands of interest from Pro-Q gel images were located on SYPRO stained gels by visual comparison. Using the Bio Rad ChemiDoc XRS as a UV light source, bands of interest

were cut and removed from gels. Excised gel bands were placed into 1.5 mL centrifuge tubes, covered with 20% acetic acid and stored at -80°C.

Protein Sequencing

Selected excised proteins will be sequenced by mass spectroscopy at the Proteomics Laboratory at The Ohio State University.

RESULTS

Growth of *Penicillium marneffei* conidia was completed in several trials as to maintain fresh stock cultures and 24 hour mold and yeast cultures. Conidia were grown as described in methods, producing cultures fluffy and white in appearance as well as producing red pigment into solid PDA medium. Figure 3.1 shows typical appearance of conidia stock cultures.

Figure 3.1 Conidia Stock Culture. 150 cc culture flask containing *Penicillium marneffei* grown for 10 days at 25°C. Powder white appearance of conidia and red pigment is visible.

Mold and yeast cultures were produced by conidial inoculation of SAB and growth for 24 hours at 25°C and 37°C respectively. At the time of cell harvesting, microscopic analysis was done to examine cell morphology and to ensure to absence of contamination. Figures 3.2a and 3.2b show photomicrographs of mold and yeast cultures.

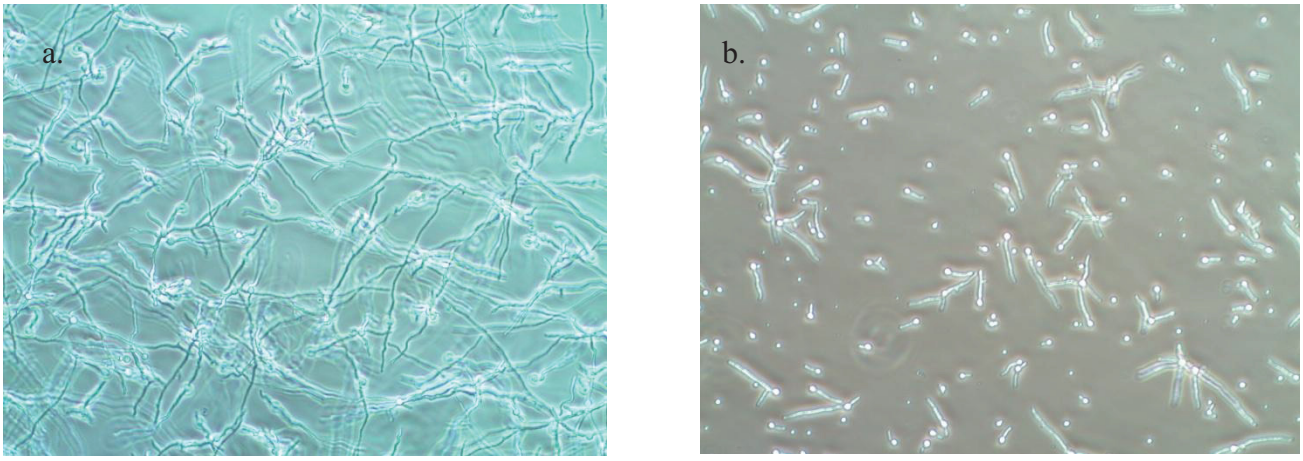
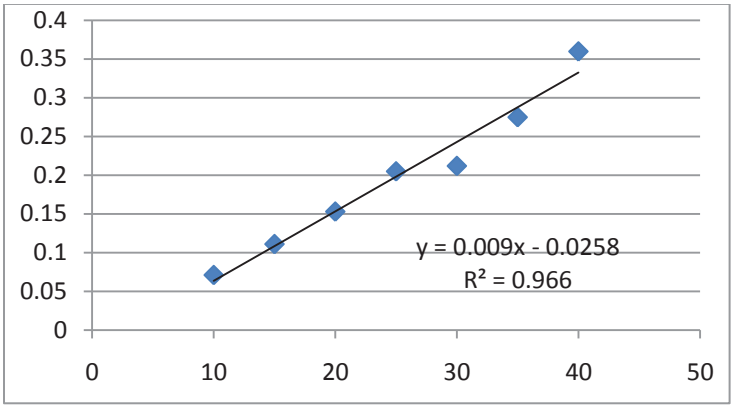


Figure 3.2 Photomicrographs of mold and yeast. a) Mold cells at 100X. Elongated, multinucleate hyphae are visible. **b)** Yeast cells at 100X. Shortened arthroconidia are shown.

Figure 3.2a shows elongated hyphal structures produced by incubation at 25°C. Due to their morphology and volume, mold cells pelleted less efficiently by centrifugation than did the smaller yeast cells. Therefore, extra flasks of mold were often grown to ensure usable amounts of cells for protein extraction. Figure 3.2b shows yeast cells after 24 hours growth at 37°C. Arthroconidiation is evident as the process of phase transition in *P. marneffei* is captured. Yeast cells were easily pelleted by centrifugation as described in methods.

Mold and yeast cell pellets were used for isolation of whole cell proteins by means of bead beating protocol as described above. Modified Bradford assays were performed to quantify protein samples. Figure 3.3a and 3.3b show typical data from a Bradford assay.

a.



b.

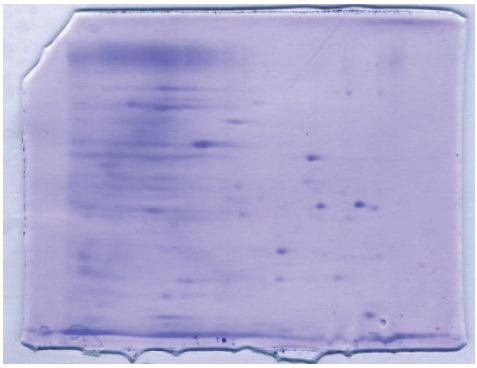
Samples				
	M1	M2	Y1	Y2
	0.21	0.472	0.615	0.661
	0.187	0.514	0.563	0.581
Avgerage	0.1985	0.493	0.589	0.621
Amount in ug	24.9222222	57.64444	68.31111	71.86667
Conc. Ug/uL	2.49222222	5.764444	6.831111	7.186667

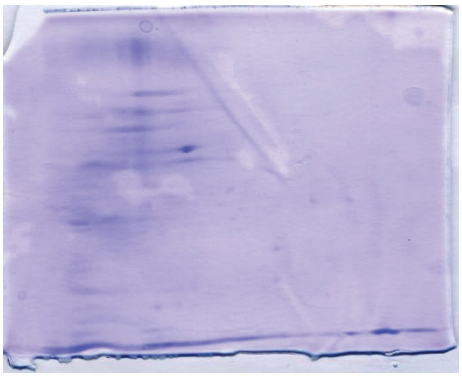
Figure 3.3 Bradford Assay. a) Standard curve of typical Bradford assay produced with Microsoft excel. Shows abosorbancies of BSA standards. R^2 values of less than 0.95 were rejected and assay was repeated. **b)** Table showing computed concentrations of unknown samples.

Concentration data obtained from Bradford assay was then used to determine the amount of sample load for gel electrophoresis.

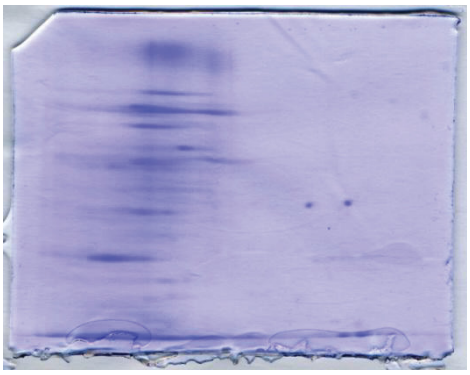
Originally, two- dimensional gel electrophoresis (2DGE) of protein samples was performed. Phosphoprotein detection was to be done via western blotting of 2DGE gels using antiphosphoaminoacids. Protein profiles produced using 2DGE were of undesirable quality for use in the western blotting technique. Figure 3.4 shows examples of 2DGE gels stained in comassie blue.

a. b.





c. d.



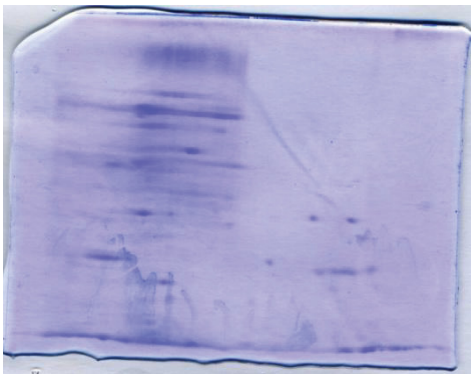


Figure 3.4 Two-dimensional gel electrophoresis. a and b) 2DGE profiles of mold protein samples. 7 cm gels, pH range 4 to 7. **c and d)** 2DGE profiles of yeast protein. 7 cm gels, pH range 4 to 7. All gels stained overnight in Comassie blue.

Poor resolution of protein spots and low throughput of 2DGE and western blotting were cause for changing methods to the use of large format one-dimensional gel electrophoresis (1DGE) and Pro-Q diamond phosphoprotein stain. The Protean II xi cell allows for up to 25 samples to be run per gel, a much higher throughput than that of 2DGE. Protein band patterns produced by 1DGE and Pro-Q diamond phosphoprotein staining were optimal for the investigation of differential phosphoproteomics in *P. marneffei* as desired in this study. Figure 3.5 depicts a one dimension 16 cm gel of both mold and yeast samples.

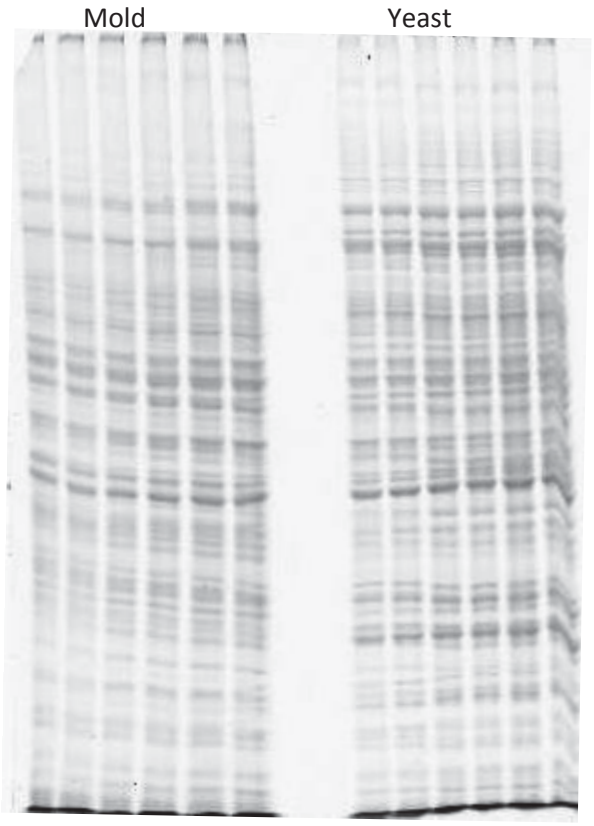


Figure 3.5 One dimensional gel of mold and yeast. Lanes 1 through 6 show mold protein samples in varying load amounts. Lanes 7 through 12 show yeast protein samples in varying load amounts. Gel was SYPRO stained overnight and imaged using the Bio Rad Pharos FX plus molecular imager.

To determine the optimal load amount for one dimensional electrophoresis, a preliminary gel was run with a gradient of concentrations as shown in figure 3.5. Samples were loaded in duplicate ranging from 15 µg to 25 µg in 5 µg increments. Load amounts of 20 µg and 25 µg of protein were then used for the remainder of the study as they produced optimal banding patterns.

Pro-Q Diamond phosphoprotein gel staining revealed differential phosphoproteomes in mold and yeast whole cell protein extract. Figure 3.6 shows a 1D gel stained with Pro-Q Diamond. Differential band patterns are evident in the gel image and are highlighted in the lane comparison graph made with Quantity One, also shown in figure 3.6. Unique yeast bands are highlighted using green arrows and double sided blue arrows depict common bands.

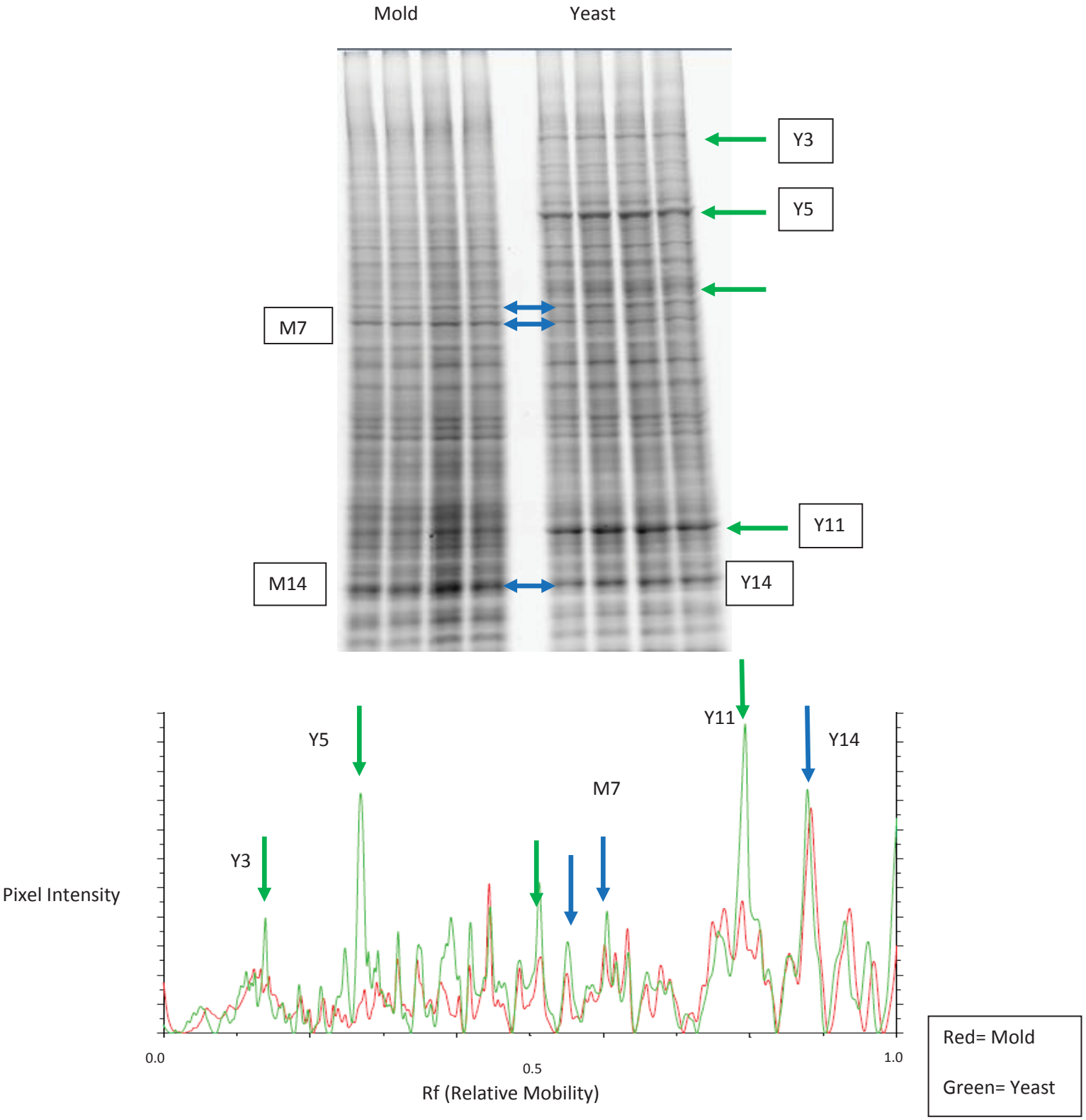


Figure 3.6 Differential phosphoproteomics of mold and yeast. Pro-Q Diamond gel image taken with Bio Rad Pharos FX molecular imager. Accompanying graph made with Quantity One, also from Bio Rad, shows differential phosphoprotein band patterns among mold and yeast proteins.

The gel shown in figure 3.6 also contained a molecular weight standard which is not visible in the Pro-Q image, as it contains no phosphoproteins. This, along with the appearance of more numerous protein bands in SYPRO stained gels, acted as a control for all Pro-Q and subsequent SYPRO stained gels as it demonstrated very little nonspecific binding of Pro-Q to phosphoprotein targets. Also shown in figure 3.6 are protein bands of interest which were generically named and selected for excision. It is important to note that these bands represent both different phosphoproteins as well as similar proteins that are phosphorylated differently in each cell state.

Upon Pro-Q imaging, gels were immediately placed in SYPRO and allowed to stain overnight in order to visualize total protein patterns for both mold and yeast. Staining with SYPRO produced more numerous bands as well as exposure of molecular weight standard.

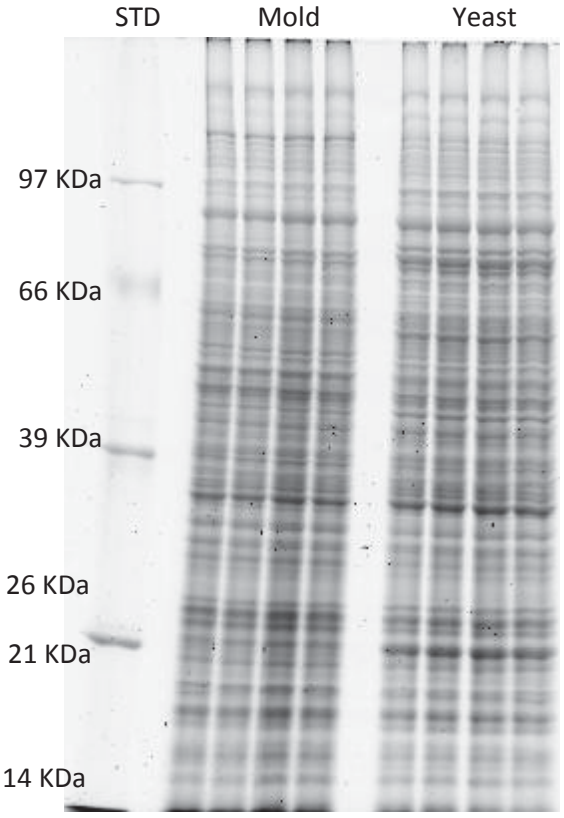


Figure 3.7 Pro-Q stained gel stained in SYPRO. 1D gel of mold and yeast protein samples. SYPRO staining reveals molecular weight standard and other protein bands not visible with Pro-Q staining alone.

Figure 3.7 shows a gel stained in SYPRO that had previously been stained with Pro-Q. SYPRO stained gels were then analyzed in Quantity One using the "lane report" tool, which displayed molecular weight data for all visible bands. This data was then extrapolated to determine the molecular weights of phosphoprotein bands of interest. Table 3.1 below shows extrapolated molecular weights for bands of interest in Pro-Q stained gels.

Protein Name	Extrapolated Molecular Weight (KDa)
Y3	112.25
Y5	82.68
M7	47.03
Y11	19.79
M14 and Y14 (common protein)	16.33

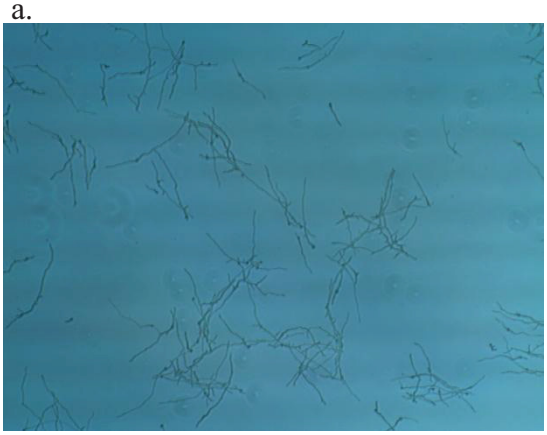
Table 3.1 Extrapolated molecular weight data

The largest protein of interest was named Y3 and has an estimated molecular weight of 112.25 KDa. A protein band common to both mold and yeast was named M14 and Y14 respectively and is estimated to be 16.33 KDa. This is also the smallest protein of interest.

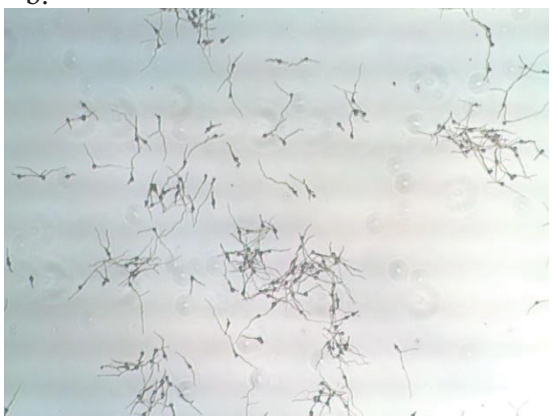
For the study of the effects of rapamycin on *Penicillium marneffei,* a rapamycin dose of 200 ng/mL was added to 24 hour cultures exactly 12 hours after inoculation. Cell growth was affected by rapamycin treatment as there was less growth per flask when compared to untreated cultures. Morphological effects were also evident in the presence of rapamycin. As shown in figure 3.8a and 3.8b, growth at 25°C was less in rapamycin treated cultures than of that in untreated cultures. Hyphal structures are much shorter in length when in the presence of

rapamycin. Growth at 37°C was more so affected than at 25°C. Figures 3.8c and 3.8d show yeast cells at 37°C. In figure 3.8c, growth has proceeded as in earlier trials of the study where arthroconidiation is evident after 24 hours of growth. In figure 3.8d, a dramatic reduction in cell growth and differentiation is evident as the presence of rapamycin allows for extremely sparse

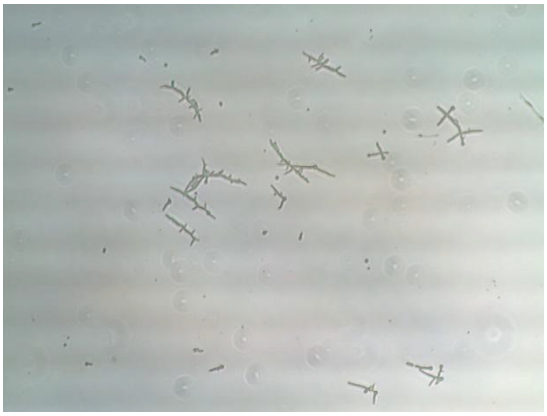
a.



b.



c.



d.

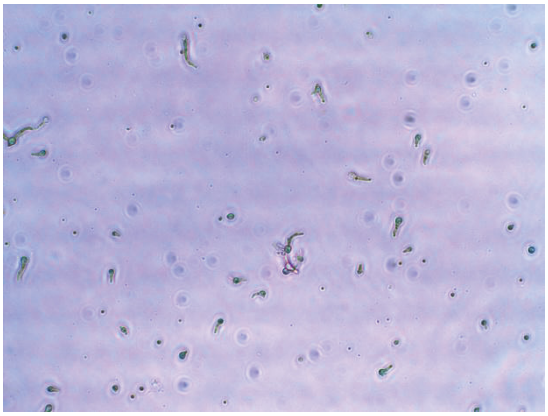


Figure 3.8 Photomicrographs of rapamycin treated and untreated mold and yeast cultures. **a)** Untreated mold culture at 40X after 24 hours of growth at 25°C **b)** Rapamycin treated mold culture at 40X after 24 hours growth at 25°C **c)** Untreated yeast culture at 40X after 24 hours growth at 37°C **d)** Rapamycin treated yeast culture at 100X after 24 hours growth at 37°C

amounts of arthroconidiation. Cells appear to be locked in a conidial-like morphology with very short and infrequent hyphae.

After 24 hours growth at 25°C and 37°C, rapamycin treated cells were harvested and pelleted. Whole cell protein was then extracted and subjected to 1DGE. Pro-Q Diamond phosphoprotein gel stain was used to analyze differential phosphoproteomics produced by rapamycin. Data is shown in figures 3.9 and 3.10 below.

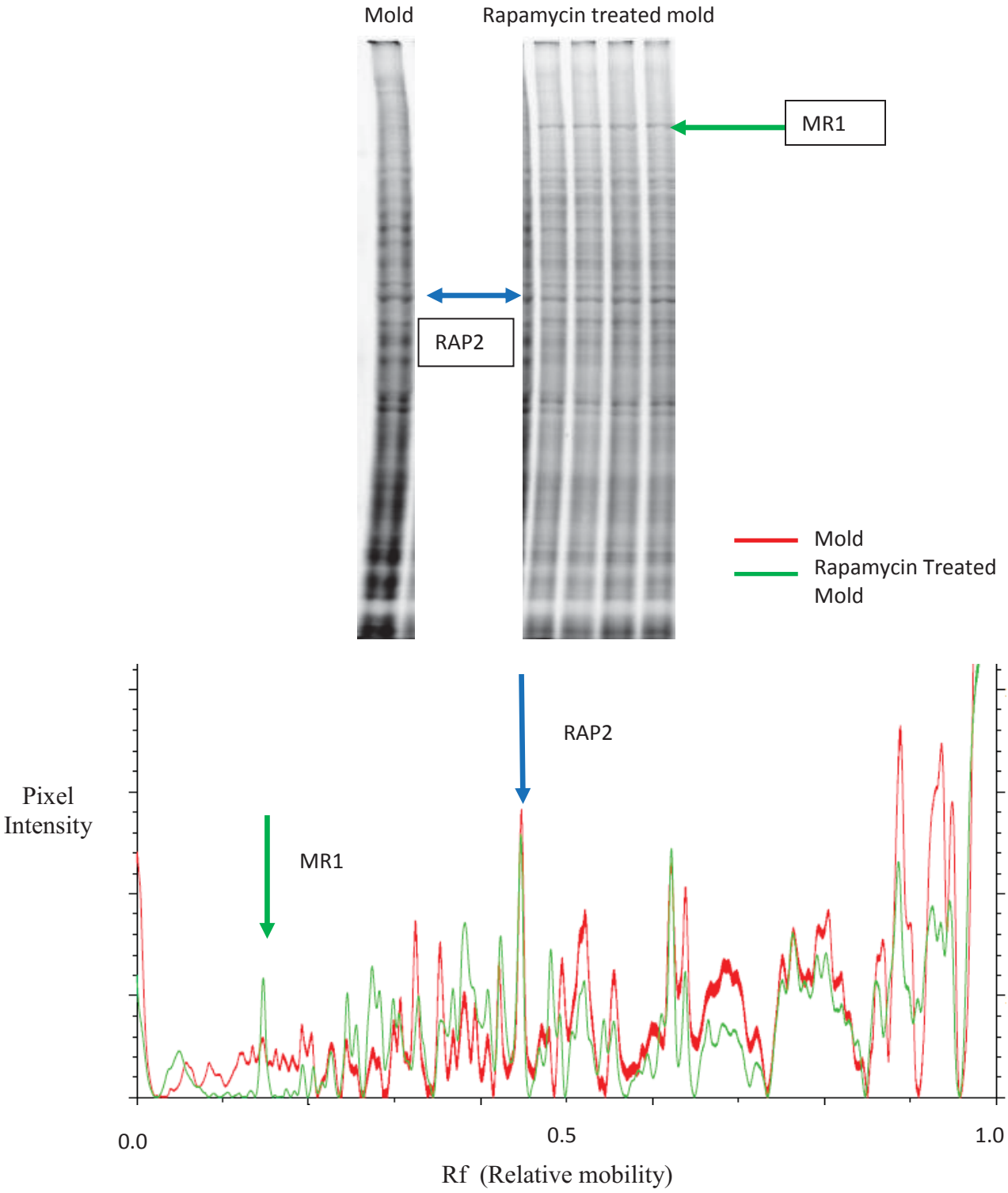


Figure 3.9 Differential phosphoproteomics of rapamycin treated mold. Pro-Q Diamond stained gel image taken with Bio Rad Pharos FX molecular imager. Graph made with Quantity One. Shows phosphoproteomic changes caused by rapamycin in mold.

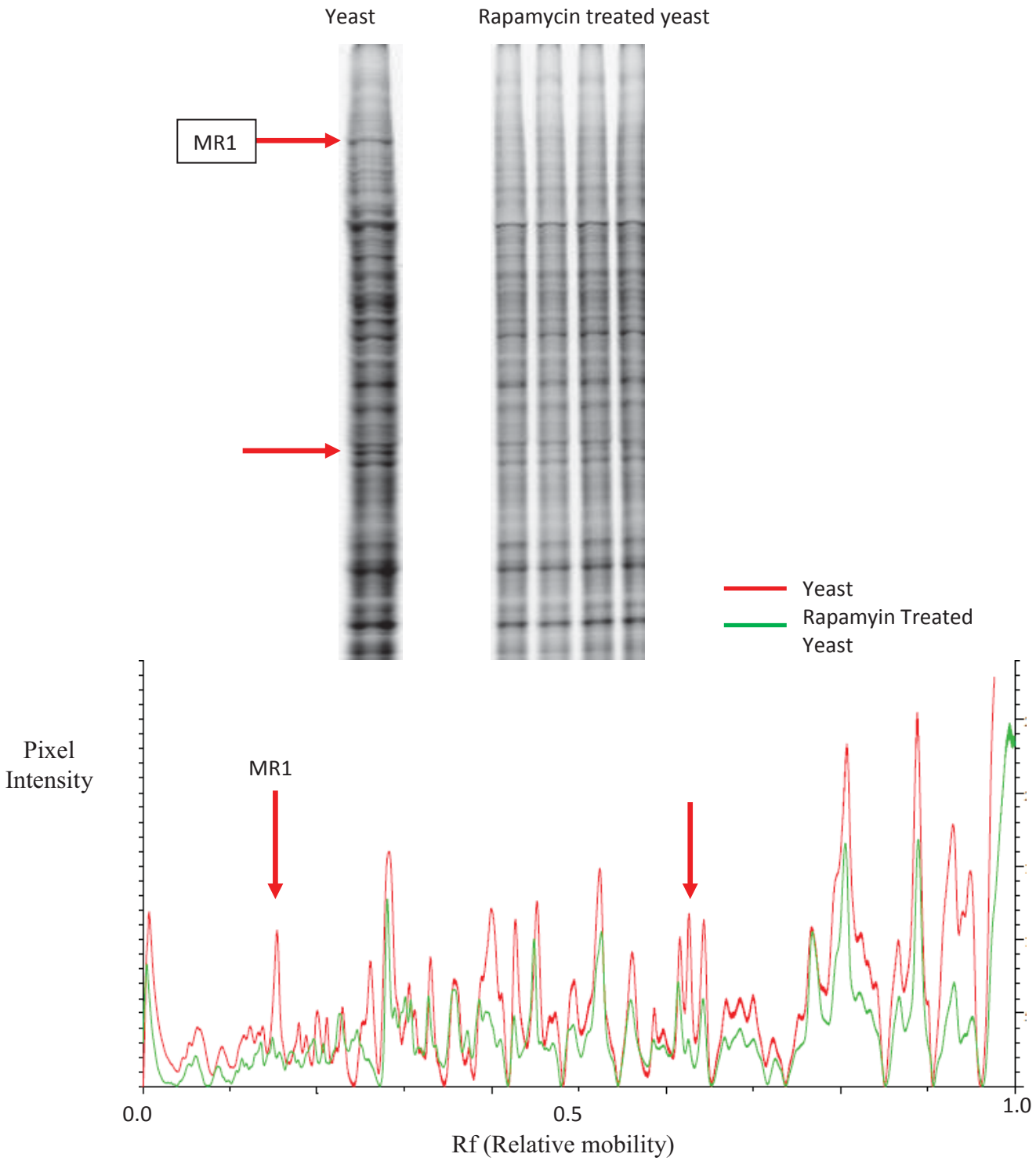


Figure 3.10 Differential phosphoproteomics of rapamycin treated yeast. Pro-Q stained gel image taken with Bio Rad Pharos FX molecular imager. Graph made with Quantity One. Shows phosphoproteomic changes caused by rapamycin in the yeast phase.

Figure 3.9 and 3.10 show phosphoproteomic changes caused by rapamycin treatment in mold and yeast cells respectively. The protein band labeled as MR1 was excised from the gel. This band appears in the same position as the formerly labeled protein band Y3 shown in figure 3.6, which was also excised.

Immediately following imaging, Pro-Q stained gels shown in figure 3.9 and 3.10 were stained overnight in SYPRO to reveal total proteome band patterns of rapamycin treated cultures.

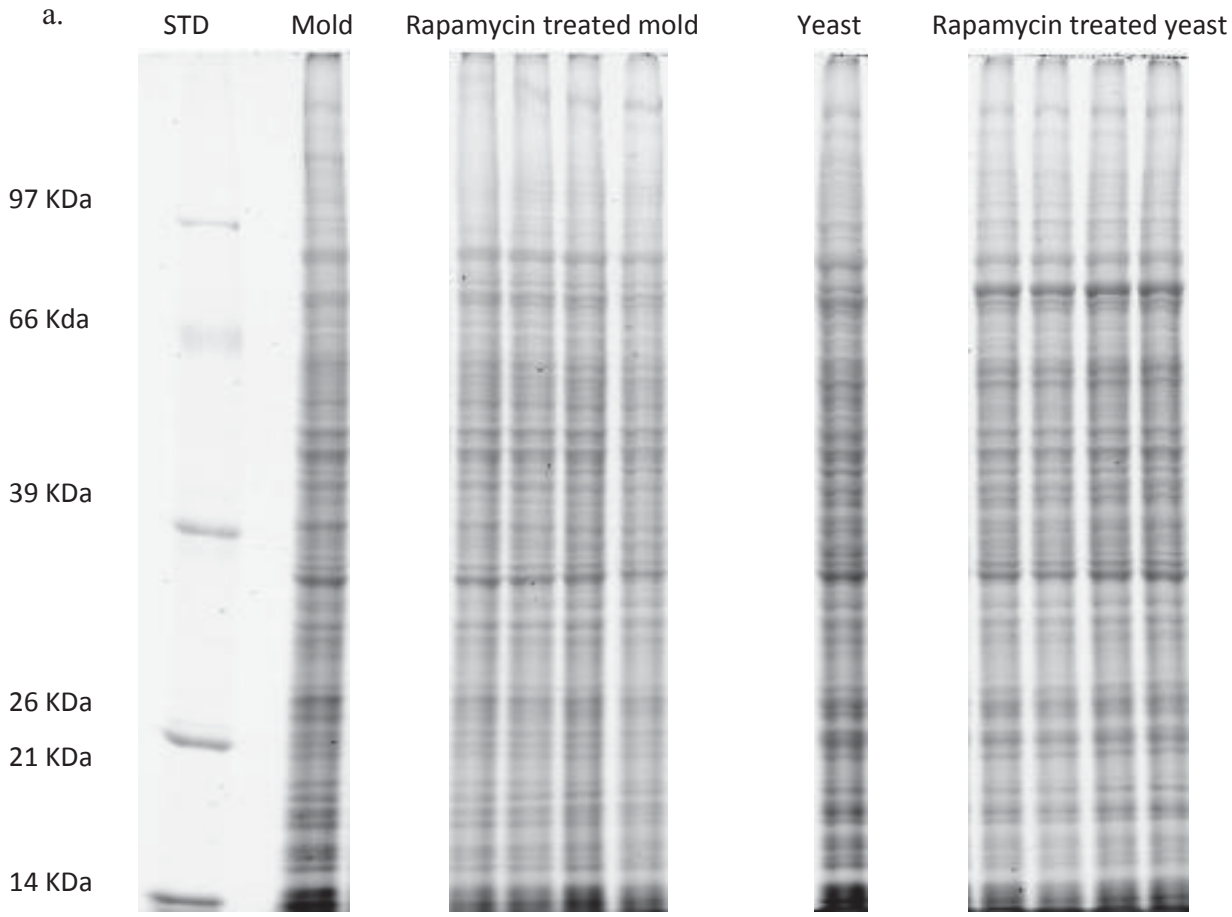


Figure 3.11a SYPRO Stained 1D Gel. 1D gel stained in SYPRO, shows presence of molecular weight standard otherwise not visible in Pro-Q stained gels.

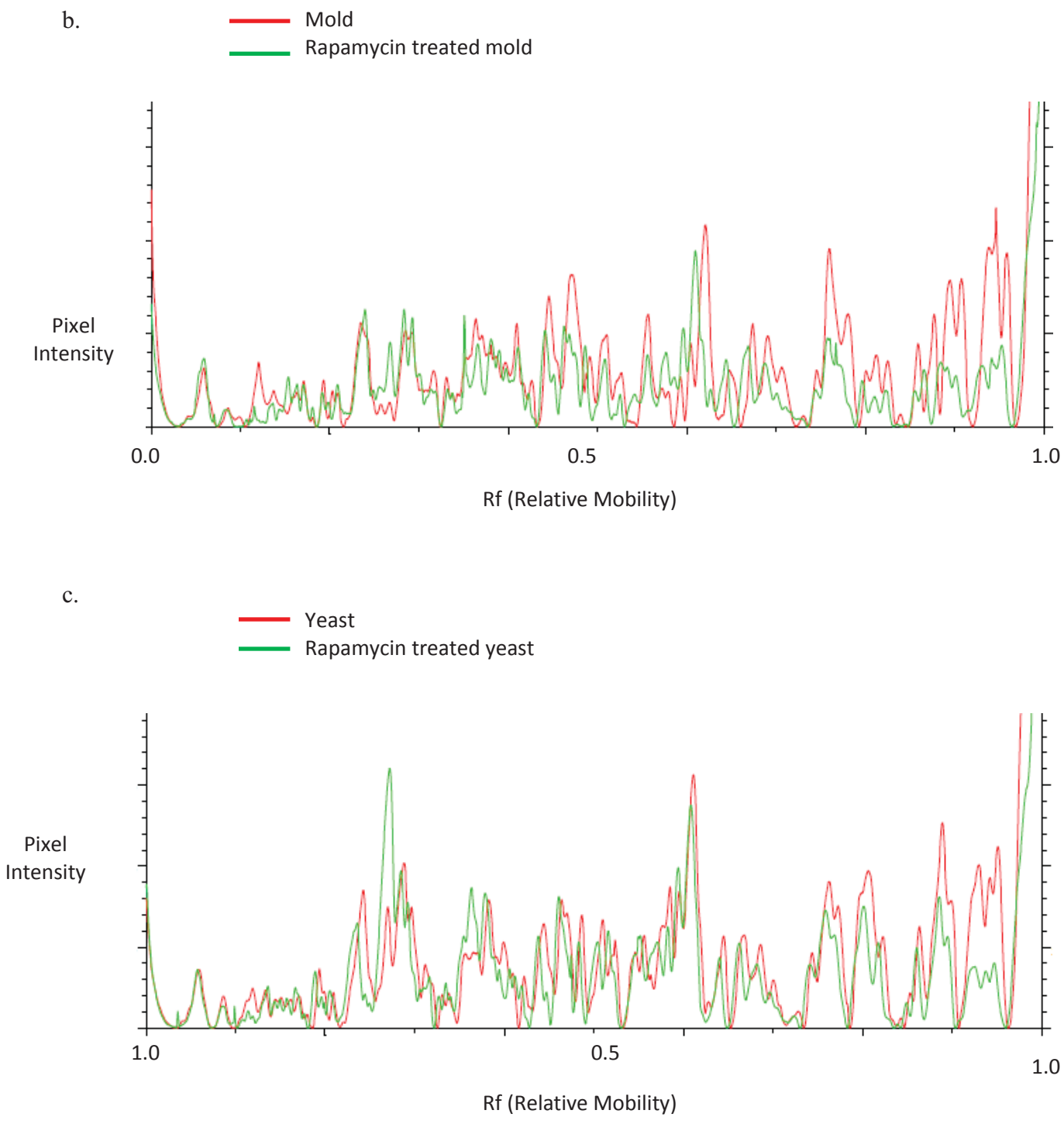


Figure 3.11b and 3.11c Lane comparison graphs. b) Lane comparison graph of untreated mold (red) and rapamycin treated mold (green). **c)** Lane comparison graph of untreated yeast (red) and rapamycin treated yeast (green).

As shown in figure 3.11, SYPRO staining revealed the presence of a molecular weight standard which is not visible in the Pro-Q stained gel image. This serves as a control for the selective staining of phosphoproteins by Pro-Q. Total protein data from figure 3.11 indicates that in addition to phosphoproteomic changes, rapamycin also produces changes in the proteome of both mold and yeast cells as quantitative differences are evident in the lane comparison graphs.

DISCUSSION

Throughout the course of this study, *Penicillium marneffei* stock cultures were maintained on 50 cc PDA flasks every 15 to 20 days. This was done to ensure fresh supplies of conidia for inoculation of larger 150 cc culture flasks for eventual growth of 24 hour mold and yeast cultures. Growth protocols for mold and yeast cells were followed from research done by Chandler et al (7). These protocols allowed for optimal cell concentrations for harvest and homogenization.

Protein isolation described in methods above, is also a protocol derived from Chandler et al (7) with the exception of the addition of phosphatase inhibitor buffer to preserve phosphoproteins. The phosphatase inhibitor protocol was derived from Kaufmann et al (13) and was made fresh for each homogenization trial. Successful isolation of phosphoproteins shown by Pro-Q staining ensures that phosphoproteins were indeed preserved during the homogenization procedure.

Initially, two dimensional gel electrophoresis was intended to be used for separation of whole cell protein extracts. This was then to be followed by western blotting for phosphoproteins with the use of antiphosphoamino acids. Two dimensional protein patterns were of undesirable quality to proceed with western blotting (Figure3.4). Initially, protein samples appeared to be too acidic as protein spots only appeared on portions of 2D gels of low pH. This problem was worked out by resuspending protein pellets into fresh MSB. The use of fresh MSB allowed for better resolution of protein spots than in previous trials but were still not of desirable quality to proceed with western blots.

Due to poor quality of two dimensional gels the decision was made to use one dimensional gel electrophoresis (1DGE) instead. There were many advantages to 1DGE including the use of less protein sample, less time per gel as the need for isoelectric focusing was eliminated, and the elimination of the western blotting technique. Staining one dimensional gels with Pro-Q Diamond Phosphoprotein gel stain became a crucial tool for the revelation of phosphoproteins in whole cell protein samples. The Pro-Q Diamond protocol was followed per manufacture's recommendations with special measures taken to ensure that Pro-Q staining was done in an environment absent of light as to avoid light contamination. Plastic containers designated only for Pro-Q staining were spray painted black to block out external light. Staining procedures were also done in appropriate areas and times when lab lights could be turned off. Imaging of Pro-Q stained gels was facilitated by the Bio Rad Pharos FX Plus molecular imager as it contains filters capable of imaging at an excitation/emission maxima of 555/580 nm. Initial 1DGE gels were stained only with SYPRO and revealed excellent separation of protein samples by the production of distinct protein bands. These results encouraged further use of 1DGE as the main tool for separating protein samples.

SYPRO stained gels of mold and yeast whole protein confirmed results obtained by Chandler et al in 2008 in which differential proteomes of mold and yeast protein extract was uncovered (Figure 3.5) (7). Differential proteomics displayed in this study of mold and yeast cells as well as photomicrographs showing distinct morphologies (Figure 3.2) served as a control ensuring that phase transition had indeed occurred throughout the 24 hour growth span. Uncovering of phosphoproteins in these same protein extracts would for the first time give insight into differential phosphoproteomics brought about by phase transition in *Penicillium marneffei.*

Pro-Q Diamond stained gels of mold and yeast protein showed differential phosphoproteomics brought on by temperature dependent phase transition in *Penicillium marneffei* (Figure 3.6). Several phosphoprotein bands were shown to be unique to the yeast phase or common between both mold and yeast. Subsequent SYPRO staining of these gels revealed a greater amount of protein bands as well as the presence of the molecular weight standard also loaded onto the gels (Figure 3.7). These results show that Pro-Q staining revealed only a population of proteins in the samples and that very little nonspecific binding occurred throughout the Pro-Q staining protocol. This marks the first time differential phosphoproteomics has been displayed as a result of phase transition in *P. marneffei.* Sequencing and identification of differential and common proteins will lend more insight into the role of protein phosphorylation as it relates to morphological changes brought about by the process of phase transition.

This study also sought out to broadly study the role of the protein kinase TOR in phase transition of *P. marneffei.* Using the drug rapamycin to render TOR ineffective, its function could then be examined on a morphological, phosphoproteomic, and proteomic level. The rapamycin concentration of 200 ng/mL was derived from work done by Torres *et al,* 2002 (22). Morphological results show that in the presence of rapamycin, phase transition is severely slowed in *P. marneffei* as yeast phase cell types produced under non treatment conditions failed to form in several trials of rapamycin treatment (Figure 3.8). Cell growth was also affected in mold cell types as rapamycin treated cultures at 25°C showed less numerous and less mature forms of mold cells. One dimensional gels stained with Pro-Q show that rapamycin treatment also affected the phosphoproteomes of mold and yeast cells. In the mold phase, treated cells display a phosphoprotein band extrapolated to be 103.87 KDa in size that does not appear in the untreated sample (Figure 3.9). This protein also appears in the SYPRO stained gel indicating that

the regulation is brought about at the post translational level. Phosphoproteomic changes were also evident in the yeast phase when in the presence of rapamycin (Figure 3.10). A phosphoprotein band prevalent in the untreated sample does not appear in the treated sample. This band was extrapolated to have a molecular weight of 109.13 KDa. This result bears great similarity to the mold band mentioned above and it is likely that they are the same protein, regulated differently by TOR in the process of phase transition.

Overall, this research has successfully revealed differential phosphoproteomics among mold and yeast cell types as a result of temperature dependent phase transition in *Penicillium marneffei.* It has confirmed the used of 1DGE in conjunction with Pro-Q Diamond Phosphoprotein gel stain as a reliable technique for studying phosphoproteomes (12). It has also shown the morphological and phosphoproteomic effects on phase transition produced by the drug rapamycin in *P. marneffei.* Sequencing and identification of excised proteins will lend more insight into the role of protein phosphorylation and TOR kinase activity in the transition from mold to yeast cell types. This in conjunction with the differential proteomic expression in phase transition previously shown by Chandler et al will add further understanding to the molecular biology of dimorphism in *P. marneffei* (7). Considering the importance of dimorphism in pathogenicity of *P. marneffei* and other dimorphic fungi alike (8), novel drug targets and treatments may arise as a result of the findings in this study.

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