Differences in TOR and Yak1 gene expression in the Mold and Yeast phases of *Penicillium marneffei*

By

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ABSTRACT

The study was conducted on the fungus *Penicillium marneffei*. *Penicillium marneffei* is endemic to south-east Asia and is the only thermally dimorphic species of the Penicillium genus. In its yeast form, it causes a systemic disease called Penicilliosis in immunocompromized individuals. It is currently treated by using moderately effective drugs like Amphotericin B. The dimorphic switch that changes the mold to yeast at 37 °C remains a mystery and could hold the key to designing effective treatments.

Dimorphism could be hypothesized to depend on turning on or off certain genes like cell division or cell growth genes. The chief cell growth gene studied for this project is TOR or Target of Rapamycin. It is investigated for differential gene expression in the Mold and Yeast phases of the *P. marneffei* cells caused by the environmental stress. Also, the cells were treated with rapamycin to inhibit TOR and notice its effect on cell growth. TOR is characterized using bioinformatics analysis. Morphological study is conducted on un-treated and treated cultures using phase contrast microscopy.

Cells are collected to extract RNA from the different samples and cDNA is synthesized. To study expression in real time, Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) is performed on the samples using Actin and Calmodulin as controls. The expression of TOR increases in the yeast phase in both un-treated and rapamycin treated *P. marneffei* cells even though the growth is severely stunted. This indicates the presence of a compensatory mechanism by which the cells try to survive the Rapamycin in the medium and remain mostly in filamentous form. Therefore, TOR could be playing a critical role in bringing about the dimorphic change and could aid to devising enhanced treatments for Penicilliosis.

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LIST OF ABBREVIATIONS

BLAST - Basic Local Alignment Search Tool

cAMP - Cyclic adenosine monophosphate

cDNA- Complementary DNA

DNA- Deoxyribonucleic acid

FKBP- FK binding proteins

kDA- Kilo Daltons

MAPK- Mitogen-activated protein (MAP) kinases

mRNA- messenger Ribonucleic acid

mTOR- Mammalian Target of Rapamycin

NCBI- National Center for Biotechnology Information

Oligo dTs- Oligonucleotides (a short sequence of deoxy-thymine nucleotides)

PDA- Potato Dextrose Agar

pI- Isoelectric Point

PI- Phosphatidylinositol

Primer F- Forward Primer

Primer R- Reverse Primer

qRT-PCR- Quantitative Reverse Transcriptase Polymerase Chain Reaction

PCR- Polymerase Chain Reaction

SAB- Sabouraud dextrose broth

RNA- Ribonucleic acid

TOR- Target of Rapamycin

UV light- Ultra Violet light

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REAGENT RECIPES

In the order of use:

PDA (Potato Dextrose Agar) PLATES

For 10/150cm^2 plates:

25 .3g Potato Dextrose Agar

650mL De-Ionized water

Must be autoclaved

SAB Broth (Sabouraud dextrose broth)

30.0g SAB

1.0L De-Ionized water

Must be autoclaved

TE (Tris-EDTA) Buffer, 1L

10 mL 1M TRIS (pH 8.0)

2 L 0.5M EDTA (pH 8.0).

Must be autoclaved

1X TAE (Tris-Acetic Acid-EDTA) Buffer (25L)

1.0L 25X TAE buffer concentrate

24.0L De-Ionized water

No autoclaving required

INTRODUCTION

I. Kingdom Fungi :

Fungi are eukaryotic organisms found abundantly in almost every environment. There are estimated to be over a million species of fungi and only about 5% of them are identified. The fungal kingdom covers a wide variety of taxa. Fungi exist in various forms like microscopic yeasts, molds and larger mushrooms. Yeasts are unicellular, asexually reproducing organisms. Molds grow as multicellular filaments. Fungi are divided into many subdivisions. Ascomycetes are one of the divisions of fungi with the largest number of species. They are monophyletic and produce diakaryotic stage and are functionally diploid (Lutzoni *et al.*, 2004). It has a specialized structure called "ascus" for sexual reproduction that produces "ascospores" or also known as fruiting bodies. The *Penicillium* thallus usually consists of hyphae and the mycelia bears the conidiophores.

II. *Penicillium marneffei*:

Penicillium marneffei is a unique pathogen. It is a thermally dimorphic fungus, the only *Penicillium* species exhibiting dimorphism. The life cycle of *P. marneffei* is broadly divided into three main phases, filamentous mold or the vegetative growth at 25 °C, conidiation or the asexual reproduction at 25 °C and unicellular yeast-like vegetative growth at 37 °C. *P. marneffei* is considered an imperfect ascomycete as no sexual cycle has been observed so far. The filamentous mold at room temperature produces a red pigment when grown for longer than 24 hours which is also currently researched. When grown at 37 °C, the fungus grows as unicellular yeasts. At 25 °C, when the conditions are favorable, the germination of uninucleate conidia occurs which produces spores. This uninucleate conidium grows to form a germ tube isotropically leading to production of

multicellular conidiophores which bear the conidia on the fully grown mycelium. The unicellular yeast growth in *P. marneffei* is similar to *S.pombe* dividing by fission to produce elongate cells. The transition of growth from multicellular hyphae to unicellular yeast is highly complex and not fully understood. Morphologically, it is observed that when placed at 37 °C, the Germinated conidia produce highly branched hyphal cells (Fig. 1). The cell and nuclear division cycles couple to produce uninucleate hyphal compartments by a process named as arthroconidiation in which the pre-arthroconidial cells separate as the cell wall material between the double septa is degraded to liberate single uninucleate yeast cells (Andrianopoulos, 2002).

The change in temperature triggers the dimorphic switch in *P. marneffei*. This transition is controlled by various regulatory genes. For example, in *Histoplasma capsulatum*, several transcriptional regulators such as W1-1, hsp60, hsp82 and YPS3 serve as regulatory switches for genes controlling temperature sensing, carbon source and iron acquisition and are believed to influence gene expression in the host environment (Ignatov & Keath, 2002). Unlike other dimorphic species, the molecular features of this dimorphic switch are not completely understood and are an active area of study. The knowledge of the key players in the molecular pathways occurring during transition will prove critical as it might shed light on the pathogenicity of this species at 37 °C. This will help in synthesizing drugs targeted to the virulent factors causing systemic infections in immunocompromized individuals.

P. marneffei was not discovered until the year 1956 from the hepatic lesions of the bamboo rat. This species is most common in Southeast Asia. The human

pathogenicity was diagnosed between 1984 and 2004 in Thailand. *P. marneffei* causes a systemic infection known as "Penicilliosis" in immuno-compromized individuals.

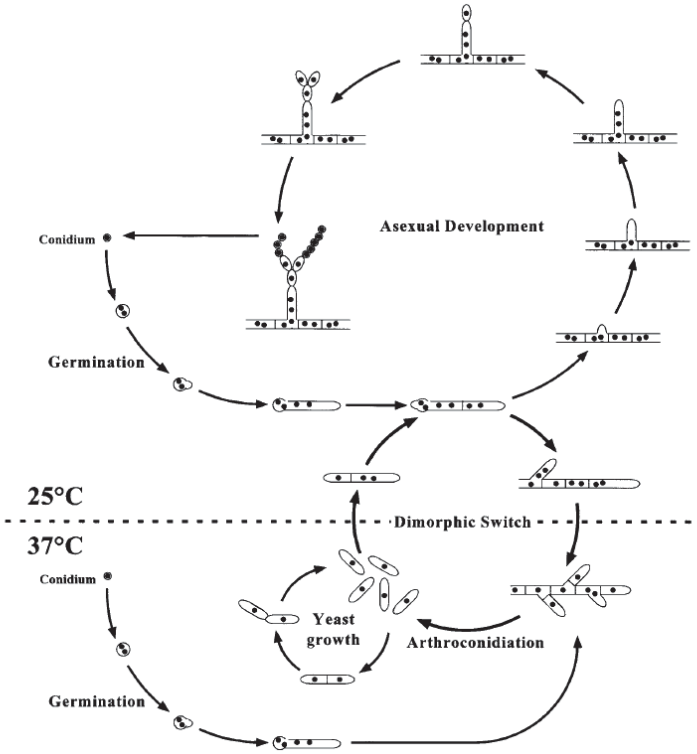


Figure. 1 Penicillium marneffeilife cycle [Andrianopoulos, 2002]

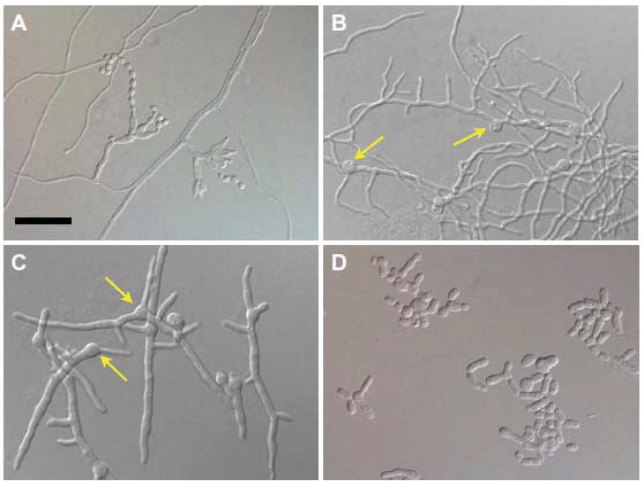


*Figure. 2 Thermal dimorphism in P. marneffei. **A** and **B** represent the mold phase at 25 °C. **C** represents the beginning of the transition and **D** shows the unicellular elongated yeast cells. [Chandler et al. 2008]*

The first case of human infection due to this fungus was found in 1973. These infections are not only found in Thailand, but other South-East Asian countries like India, China, Malaysia and Taiwan (Vanittanakom and Cooper, 2008). This infection can be lethal with a varying degree of severity. The symptoms are mainly fever, weight loss, skin lesions, generalized lymphadenopathy, hepatomegaly, ulcers and anemia depending upon the immunity of the host individual. It can usually be treated with antifungal agents. Studies have shown that the yeast form of *P. marneffei* infests at the body temperature in the patients suffering from Penicilliosis. At large, this fungus is mainly found endemic to South-East Asia and neighboring areas infecting the people suffering from HIV infection, AIDS, or low immunity because of surgery or other factors. The basic treatments for penicilliosis

Conclusions drawn from various studies imply that the transcription regulators and the genes responsible for dimorphism or cell cycle events like *abaA, stuA*, or *tupA* in other fungal species do not regulate the dimorphic transition in *P. marneffei*. This makes presence of a completely separate mechanism controlling dimorphism highly apparent and so far obscure and mysterious. One can however deduce that study of the differential expression of proteins in the yeast and the mold phases might give an insight to the changes occurring during phase switching. Liu *et al.*2007*,* used subtractive hybridization to screen 500 genes using forward and reverse-subtracted cDNA libraries and cDNA array dot blotting and came up with 43 differentially expressed genes in the yeast and mold phases of *Penicillium marneffei.* It was noteworthy that these genes included genes involved in cell wall synthesis, signal transduction, cell cycle, substance transport, general metabolism, stress response genes and other functional categories broadening the

research horizon for further investigation. The protein profiles of various proteins studied by Chandler *et al*. (2008) using protein quantification, 2 dimensional gel electrophoresis, mass spectrometry, PCR and DNA sequencing revealed various proteins ranging the in pI 5-8. Many of these proteins like general metabolism, energy production, signal transduction, ribosomal structure etc. were common to both yeast and mold phases but many were unique to just one phase. Certain proteins however like the heat shock and stress related proteins were up/down-regulated in one phase. Cell growth gene regulators such as Y544, translation initiation factors for eIF2b and Y552, a small monomeric RAS GTPase Rrs1 for spore germination were upregulated. Enzymes involved in the chitin synthesis for the cell wall are currently being investigated for differential expression in the different phases as well as the virulence of this species. Reviewing all the previously done studies indicate that the key to studying this fungus is via exploring the differential expression of various proteins which might be involved in the dimorphic switching from a non-pathogenic to pathogenic form (Xi *et al*. 2007).

III. Dimorphism and Virulence:

Amongst the several dimorphic fungi like *Paracoccidioides brasiliensis, Histoplasma capsulatum, Candida albicans and Ustilago maydis,* there is one common factor, virulence. All these thermally dimorphic species are pathogenic and cause a variety of diseases. However, the infected samples from patients all contain the dimorphic form that grows at 37 °C. Virulence and dimorphism seems to be directly related and these species have evolved to be pathogenic in only one form. The dimorphic switch occurs once the species is subjected to environmental stress. This morphological flexibility appears to be a key contributor to virulence. Two major growth related

pathways, MAPK and cAMP are investigated of crossing over and bringing about the cascades that regulate the dimorphic switch (Sánchez-Martínez *et al.*, 2001). Studying the growth regulator genes could provide useful insight into this process.

IV. Rapamycin and Target of Rapamycin (TOR)

Given all the studies done so far, dimorphism could be hypothesized to depend on turning on or off certain genes like the cell division genes. Cell growth is highly regulated depending on the nutrition availability, temperature, stress situation and cell proliferation. Growth means increase in cell mass which requires the positive regulation of anabolic processes and negative regulation of catabolic process. In budding yeasts, the growth is polar and cells have to be laid only in one direction. The chief cell growth gene studied for this project is TOR. TOR, also called Target of Rapamycin is a cell growth, proliferation and motility regulator. It is a serine/threonine protein kinase.

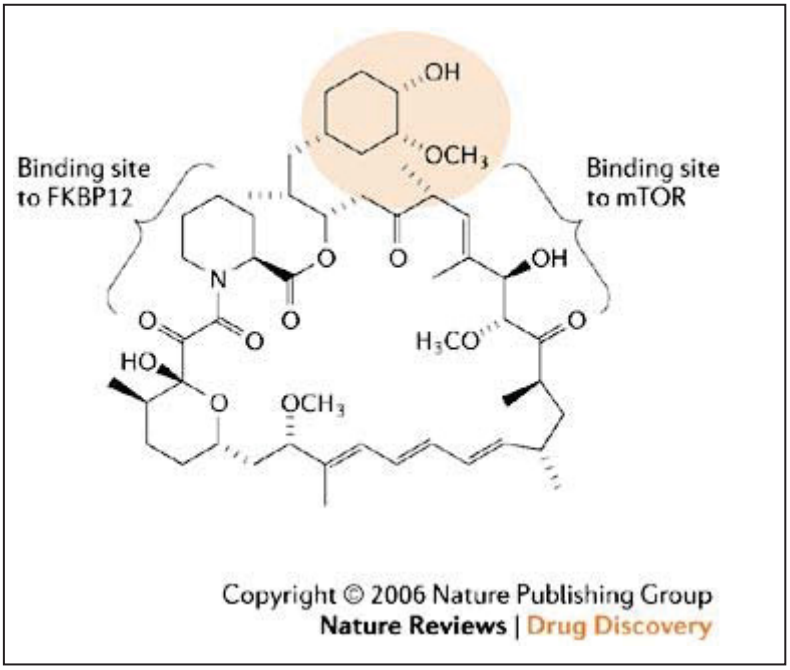


Figure 3. The structure of Rapamycin

http://www.nature.com/nrd/journal/v5/n8/box/nrd2062_BX2.html

To study Target of Rapamycin Kinase, it is important to indulge in exploring the characteristics of Rapamycin. Previously called Sirolimus, Rapamycin was isolated from a soil sample collected from Easter Island secreted by a strain of *Streptomyces hygroscopicus* in early 1970s. The culture of this strain demonstrated antifungal as well as anti-tumor and immunosuppressive properties (Cruz, 1999). The mechanism of rapamycin is as follows, TOR interacts with FKBPs, a family of intracellular FK binding. These intracellular binding proteins are collectively referred to as immunophilins which function as cis/trans peptidylprolyl isomerases (PPIase). Rapamycin binds to its respective immunophilins to inhibit PPIase activity by forming a tertiary complex with calcineurin, a Ca^{2+} calmodulin-dependent serine/threonine phosphatase, causing its inactivation in mammals. Fungal TOR has been identified to be present as TOR1 and TOR2 in yeast encoding for the protein complexes TORC1 and TORC2 (~280kDa) both having shared functions on temporal cell growth by translation initiation) and spatial cell growth by actin cytoskeleton organization (Fingar and Blenis, 2004). Its counterpart, the Mammalian TOR (mTOR) is a 289-kD protein with a C-terminal 600-amino-acid domain, homologous to many PI kinases. It was the search for the intracellular target of the sirolimus which was found to be the FKBP complex that led to the discovery of the target of rapamycin (TOR) as a critical cell growth and proliferation regulator (Sehgal, 2003).

TOR is an evolutionarily conserved proteins kinase that governs integration of nutrient signals and growth factors in eukaryotes. It is also called FRAP (FKBP12-rapamycin-associated protein), RAFT (rapamycin and FKBP12 target), or RAPT (rapamycin target), or SEP (sirolimus effector protein). It is known to act by targeting a

ribosomal protein S6 kinase and eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs). It is capable of environmental sensing of growth resources to regulate cell growth by controlling protein synthesis. mTOR has been identified and cloned from human, rat, and mouse. Structurally, it is a large 290 kDa protein, which is evolutionarily conserved member of the phosphatidylinositol 3-kinase (PI3K)-kinase-related kinase (PIKK) superfamily. The carboxyl or the COOH-terminal kinase domain with lipid kinase homology functions as a serine/threonine protein kinase *(*Fingar and Blenis, 2004). The TOR signaling pathway is unconventional and non-linear. It usually functions like any other serine/threonine kinase, i.e. by phosphorylating the "-OH" groups of the serines and threonines of various proteins. It contains a rapamycin binding domain, a catalytic domain, and a FATC (FRAP- Fk506 binding protein 12-rapamycin associated protein) domain at the C-terminus.

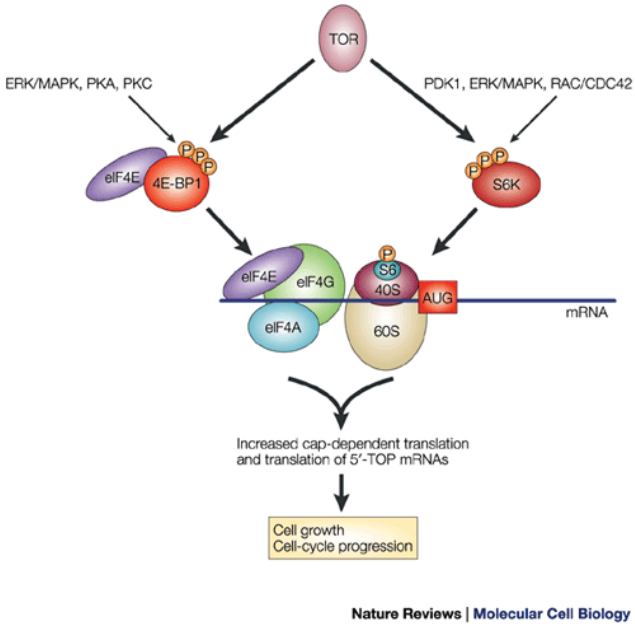


Figure 4. TOR mechanism.

http://www.nature.com/nrm/journal/v5/n5/fig_tab/nrm1365_F5.html

They also have a nutrient sensing checkpoint capacity. The TOR signal mRNA encodes the proteins necessary for cell growth and autophagy inhibition. In response to presence of nutrients, it phosphorylates 4E-BP1 and S6 kinase (Sk6). As shown in Fig 4, this initial phosphorylation leads each molecule for further phosphorylation. TOR phosphorylation releases the translation initiation factors from its repressors and enables protein sysnthesis required for cell growth. Which is why, the inhibition of mTOR-dependent signaling in cancer cells should mimic nutrient and growth factor deprivation, limiting cell growth and conferring a significant proliferative disadvantage (Tee & Blenis, 2004).TOR also works amongst the MAPK pathway through the PI-3K protein kinas B (PKB) pathway by responding to the growth factors and stimulating translation by activating p70S6 kinase (Inoki *et al*. 2005). This property could be used to target specific TOR kinases and prevent cell cycle growth the pathogenic form of *P. marneffei* causing systemic infections in immunocompromized patients. To establish a strong foundation for this study, bioinformatics analysis was done on the TOR sequence found in *P. marneffei*. BLAST, Multiple Sequence Alignment, Phylogenetic Trees, and structural analysis confirmed the presence of this protein in this fungus. The analysis also showed the phylogenetically, *P. marneffei* is closest to a filamentous fungus, *Talaromyces stipitatus* (Quest poster, unpublished data).

V. Actin and Calmodulin:

Actin is a housekeeping gene found in all eukaryotes. It is a major part of the cytoskeleton. In yeasts, it plays critical role in morphogenesis in bud growth, mediate transport of membrane bound vesicles, cell surface growth, spatial organization, polarity and cell wall formation along with chitin. Mostly, actin is present as a single gene often

expressed in different isoforms (Drubin *et al*., 1988). It is highly conservative and therefore a reliable reference gene to normalize gene expression. Another reference gene, along with actin, is calmodulin or Calcium Modulated Protein. Calmodulin assists in cellular processes like metabolism, apoptosis, intracellular movement and specifically cell polarity development. During cytokenisis in yeast, it forms crescent shaped structure in an unbudded cell through the structure between the mother and daughter cell. The localization of calmodulin is similar to actin during kinetochore formation of the microtubules and a polarized protein like actin (Sun *et al.*, 1992). This is why it can be used as a reference gene. Both actin and calmodulin are expressed consistently in all conditions.

VI. Yak1 Kinase

Yak1 was initially identified to be a one of the genes inhibiting cell growth and working against the growth promoter A kinase in a parallel pathway if over expressed in yeast by phosphorylating modules involved with growth related gene in the nucleocytoplasmic area. It is usually activated due to environmental stress, like nitrogen deficiency or cell cycle arrest in G1 phase and maintains transition to the stationary phase by balancing synthesis and decay (Garrett *et al*., 1991). However, over the years, research has further shed light on it other aspects. The transcriptional analysis also showed that it was essential to up-regulate some hypha-induced genes to initiate filamentation in *Candida albicans* and disrupts its yeast to hypha transition and in the formation of biofilms in *Candida glabrata*. They also regulate the genes engaged in mediating cell-to-cell interaction by encoding adhesions (Goyard *et al*. 2008). Due to the possibility of cross over between the Protein Kinase A and cAMP pathways, it is appealing to

investigate the possibility of its participation in phase transition from Mold to Yeast in *P. marneffei.*

REAL TIME or QUANTITATIVE RT-PCR:

Real Time Polymerase Chain Reaction or Quantitative PCR is a technique devised to convert the target mRNA to cDNA, amplify it and at the same time quantify it in real time by adding a dye or dye-labeled probe along with the Reverse Transcriptase. This enables us to see the minute changes occurring in gene expression, much easier than on a DNA gel by measuring the amplified product at each PCR cycle (Bustin *et al.* 2009). It detects the product in real time as the fluorescent dyes intercalate with the double stranded cDNA in every cycle. The dye used in this technique is Syber Green, which is an asymmetrical cyanine dye used as a nucleic acid stain as it binds to DNA. The resulting DNA-dye-complex absorbs blue light (λ_{max} = 488 nm) and emits green light (λ_{max} = 522 nm) which is a lot brighter than ethidium bromide and is detected by the machine (Bustin *et al.*, 2010). mRNA is copied to cDNA by reverse transcriptase using either the oligo dT, random primers or specific primers are used. A reverse transcriptase with an endo H activity is used. This removes the mRNA allowing the second strand of DNA to be formed. A PCR mix is set up which includes a heat-stable polymerase (such as Taq polymerase), specific primers for the gene of interest, deoxynucleotides (dNTPs) and a suitable buffer. Like the regular PCR, the denaturing of the cDNA occurs at more than 90°C separating the two strands. For the specific primers to anneal, the sample is cooled to 50°C to 60°C. The primers sites should be less than 200 bp for real-time PCR. The Taq DNA polymerase binds and extends from the primer to the end of the cDNA strand. The real-time machine (ICycler® from BioRad) containing a sensitive camera that

monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction is connected to a computer and software needed to run the real time PCR machine in real-time mode (file:*///F:/Research%20Methods/Thesis/qPCR/realtime-home.htm).* An earlier method used to quantitate the cDNA was the **standard curve method.** These days, the most common qPCR method used is the Relative Quantitation (RQ) method which uses Comparative C_T to determine the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample which could be an untreated control or a sample at time zero in a time-course study.

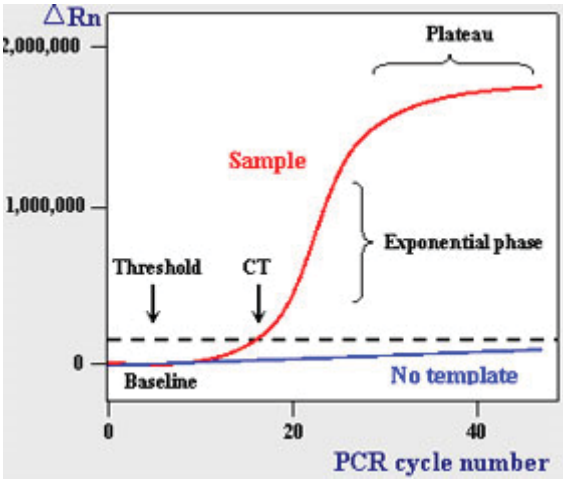


Figure 5. The Real Time PCR curve. [http://www.rt-pcr.com/]

The amplified cDNA is quantified by various methods like the determining the *threshold cycle* (Ct), *crossing point* (Cp), and *take-off point* (TOP). Without using the standard curves, RQ provides accurate comparison between the initial levels of template in each sample without requiring the exact copy number of the template. There are two types of quantitative real-time PCR: absolute and relative. A **Baseline** is the initial cycles of PCR in which there is little change in fluorescence signal. An **amplification plot** is the plot of fluorescence signal versus cycle number in which reactions are characterized by

the point in time during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed (Real-Time PCR: Understanding C_T,2008). A **Threshold** is the level of ΔRn—automatically determined (or manually set above the baseline and lower than the exponential growth region of the amplification curves) by the SDS software—used for C_T determination in real-time assays. The Ct is influenced by the concentration of the target.

PURPOSE OF STUDY

The main aim of this project is to analyze the two different forms of this temperature dependent fungus at a morphological and molecular level to understand its growth mechanisms and pathogenecity by investigating TOR expression at the mold and yeast phase of *Penicillium marneffei* using **R**everse **T**ranscription **P**olymerase **C**hain Reaction (RT-PCR) and Real-Time or **Q**uantitative **R**everse **T**ranscription Polymerase **C**hain Reaction (qRT-PCR)

HYPOTHESIS

- TOR and YAK are differentially expressed in the Mold and Yeast phases of *Penicillium marneffei.*
- Using actin and calmodulin as control genes, the normalized expression of TOR decreases with the addition of rapamycin.
- TOR plays a critical role in the dimorphic switch from Mold to Yeast in *Penicillium marneffei.*

Experimental Design:

- Conduct bioinfomatics analysis on the already given TOR sequences in the NCBI database.
- Grow *P. marneffei* strain F4 as solid cultures for 10 days. Harvest the conidia on the 11th day and grow two sets of liquid broth cultures each for 25 °C (Mold) and 37 °C (Yeast). At each temperature, treat one set with Rapamycin and leave the other un-treated (control). Examine the cultures morphologically at different time points using phase contrast microscopy.
- Collect both treated and un-treated cells for both the temperatures (Yeast and Mold phases) at 24 hours to extract good quality RNA in workable concentrations using the Qiacube and clean it using DNase treatments for high purity ratios. Synthesize cDNA from the extracted RNA using Oligo dTs and amplify it using gene specific primers.
- Determine whether there is a difference between RT-PCR product and PCR product of the genomic DNA to ensure NO-contamination/specificity of the primers and work out the best annealing temperatures for the designed gene specific primers by conducting a gradient PCR.
- Optimize the qRT-PCR procedure by conducting a standard curve for Calmodulin and conduct a qRT-PCR comparing Mold/Yeast and Mold-RAPAMYCIN/Yeast-RAPAMYCIN expression at 24 hours for TOR and Yak (only Mold/Yeast) and work out the best control (Actin/Calmodulin).

MATERIALS AND METHODS

Growing *Penicillium marneffei* cultures:

The culture plates were swabbed in a biological safety hood. It was imperative that the hood's UV lamp was turned on for 15 minutes before and after the experiment. Two large Corning 150cm^2 polystyrene canted neck flasks were poured with Potato Dextrose Agar (PDA). A *P. marneffei* strain F4 (obtained from Chiang Mai University, Thailand and maintained at Youngstown State University) stock PDA plate, not more than 15-20 days old was used. A sterile hemostat clamp flamed with 80% ethanol was used to clamp a sterile cotton swab. The cotton end of the swab was dipped in sterile water and the wet swab was used to swab a small area on stock PDA plate. Once the tip of the swab turned black, it was carefully taken out and the new PDA plate was swabbed with long gentle strokes without scraping the agar. The plates were labeled on the agar side and were incubated agar side up at 25 °C for 10 days.

Harvesting the conidia and broth inoculation:

After 10 days incubation period, in a UV sterilized biological hood, 15mL of sterile water was added to the PDA plate and rock it back and forth. Using a cell scraper gently scraped the mold containing the conidia off the plate in circular motions. Transfer the conidia solution using a 25mL serological pipet to a 50mL conical tube fitted with a sterile filter and sealed the tube with parafilm. Centrifuge the tube at 1,000 RPM speed at 4°C for 30 seconds. Poured another 5mL of sterile water and re-scraped the flask for any residual conidia and transferred it again to the 50mL conical tube with a filter to be centrifuged again at 1,000 RPM speed at 4°C for another 30 seconds in a Beckman GPR centrifuge. Resuspend the conidia pellet and combined all the conidia solutions to have

one single stock conidia solution. To determine the conidia concentration in solution, 100µL of stock solution was taken and added to 900µL sterile water in a 1.5mL eppendorf tube. This was the 10^{-1} dilution. From this dilution, took 100µL of solution and added to 900µL sterile water to make the 10^{-2} dilution. Loaded 10µL of this dilution on each side of the grid of a haemocytometer and let it sit for about 10 minutes before counting. 4 corners of each big square (16 squares total) were counted using an Olympus Bright Field microscope and repeated on the other side to average these two numbers. 10^4 is the haemocytometer constant. To calculate conidia concentration the following formula was used, Conidia/mL = (average counted)$(1x10^2)(1x10^4)$.

To find the total amount of SAB (Sabouraud Dextrose Broth) that can be inoculated using the collected conidia stock $C_1V_1=C_2V_2$ was used, where C1= determined conidia per mL, V_1= volume of stock solution (estimated from the conical tube markings), C_2= $1x10^7$ conidia per mL (this is the required concentration). For this experiment, 50mL SAB cultures in 250mL Erlenmyer flasks were inoculated and to ascertain the volume of stock required to inoculate each flask, $C_1V_1=C_2V_2$ was solved for V_1, where C_1= determined conidia per mL, C_2= $1x10^7$ conidia per mL, and V_2= 50mL. For the experiments, every time about 4 flasks for 25 °C (Mold) and 8 flasks for 37 °C (Yeast) were inoculated. After calculating the volume of the inoculums for all the mold and yeast cultures (which are the same in every way, except they are grown at different temperatures) the inoculum was poured into 15mL conical tubes and centrifuged at 3,400 RPM at 4°C for 15 minutes. The supernatant was discarded and the pellet was resuspended in SAB. From the inoculated media, 50mL was poured into each 250mL Pyrex Erlenmyer flask. These

flasks were labeled and placed in the BOKEL-GRANT scientific incubators set at 25 °C and 37 °C respectively.

Rapamycin treatment:

The Rapamycin stock of 2µg/mL from Sigma in 90% ethanol and 10% Tween stored at -20°C was added after 12 hours to half of the Erlenmyer flasks at each temperature with 200ng of Rapamycin per mL of broth (Torres *et al*., 2002) The other half of the flasks were kept un-treated as control.

Morphological Analysis:

Wet mounts were prepared with a drop of culture from each flask and were observed using an Olympus inverted phase contrast microscope and pictures were taken using an SPOT- Idea 5 MP color mosaic (260461) camera and analyzed using a SPOT-Idea software version 4.6 under 40X, 100X, 200X and 400X magnifications. Mounts from both Mold and Yeast cultures (Control and Rapamycin treated) at each time points were compared. The control cultures were analyzed at 12, 24, 48, 72, 96 and 120 hours. The Rapamycin treated cultures were analyzed at 12, 24 and 120 hours.

Cell Collection:

The cells from both the Mold cultures (25 °C) and Yeast cultures (37 °C) were collected at 24 hours and 120 hours from the control and Rapamycin treated flasks. The liquid cultures were poured in 50mL Sorvall tubes and spun after balancing at 15,000 RPM in a Sorvall centrifuge chilled at 4°C for 20 minutes. After the first spin, the supernatant broth was discarded from all tubes and they were balanced using 1X TE (Tris-EDTA) buffer and centrifuged again for 20 minutes. It was imperative that the tubes were balanced for next two spins and the pellets were saved each time. After the third spin, the thick pellets

were resuspended using 1X TE buffer and transferred to 2mL screw cap microcentrifuge tubes. These tubes were then centrifuged in an Eppendorf microcentrifuge cooled to 4°C at 15,000 RPM for 15 minutes until all the resuspended pellets was transferred to the respective 2mL screw cap tubes by discarding the supernatant. The pellet size was kept about $1/5^{th}$ of the screw cap tube containing about 300-500mg of cell pellet. During the entire process, everything was kept on ice. After the spins, the screw cap tubes containing the cell pellets were labeled and stored in -80°C freezer.

RNA extraction and the DNase treatment:

Retrieve and thaw the required number of sample tubes from the -80°C. About $1/5^{th}$ of the tube, containing approximately 3×10^6 cells were used. Too many cells lead to protein contamination and poor RNA purity ratios. To isolate the RNA, reagents from the RNeasy mini kit from Qiagen were used. The working area was cleaned by using 70% ethanol and RNaseZap from Ambion. To lyse each sample, the lysis buffer was prepared by adding 600µL of RLT from the kit and 1% of that volume, 6µL of β-mercaptoethanol was used. An equal amount of 0.5mm acid-washed glass beads as the cell pellet were added to each screw cap microcentrifuge tube. To this, the prepared lysis buffer was added. The fungal cells were lysed using mechanical disruption by bead beating the sample tubes four times in a mini bead beater from Biospec for 1 minute each by keeping the tubes on ice for 1 minute in between the bead beating. Immediately after bead-beating, the sample tubes were centrifuged in an Eppendorf microcentrifuge set at 4°C for 5 minutes at 10,000 g (1 RCF = 1 g). The pellets containing the cell debris were discarded and the supernatant containing the cell lysate was carefully pipeted to new 1.5µL eppendorf tubes, which were re-centrifuged to ensure no pellet contamination and

clean supernatant. Finally, the supernatant was transferred to 2mL sample tubes from Qiagen and were kept on ice while setting up the QIAcube (a self-operating fully automated sample prep machine from Qiagen for DNA/RNA/Protein extractions and other treatments). The machine was first wiped down with 70% Ethanol and RNaseZap from Ambion to make sure that everything stays RNase-free. Depending on the number of samples, the QIAcube was set up by putting the rotor adapters (with the RNeasy spin column, and 1.5mL collection tube in the right spots) were placed carefully in the right slots in QIAcube's centrifuge as instructed in the user manual. The 2mL sample tubes containing the cell lysate were placed in the designated slots. The reagents required for the extraction (70% ethanol, RPE, RWI and RNase-free water) were placed in the reagent tray with the caps removed. RPE and RW1 were supplied from the RNeasy mini kit. On the workbench, the DNase mixture from the Qiagen RNase-Free DNase Set (containing the lyophilized, RNase-free DNase I enzyme and buffer RDD) were placed in the right amount in slot A. After double checking the loading, the machine was turned on and the operating instructions were followed by using the protocol "RNA extraction with DNase treatment". The machine took about 45 minutes to an hour depending on the number of samples (maximum 12).

RNA quantification:

The extracted RNA was retrieved from the QIAcube in the 1.5mL collection tubes and were immediately kept on ice. The RNA was quantified using the BIO-RAD SMART SPEC spectrophotometer and a sterile BIO-RAD cuvette 45µL RNase-Free water and 5µL RNA). The samples were blanked using RNase-free water and the concentration (in µg/mL) and A_{260}/A_{280} purity ratio values were determined.

Second DNase treatment:

A second DNase treatment was conducted to ensure no genomic DNA or protein contamination. Keeping everything on ice or a cold block, 8µL of the collected RNA from each sample (regardless of the concentration) was added to a 0.2mL PCR tube along with 1µL DNase enzyme and 1µL of buffer RDD from the Qiagen RNase-Free DNase Set. This 10µL solution was incubated at room temperature (25 °C) for 15 minutes. 1µL of stop solution (50mM EDTA) was added to each sample and the tubes were again incubated at 70°C for 10 minutes in a BIO-RAD thermocycler. This entire 11µL solution was then used for the following cDNA synthesis.

Table 1. **Primer Information**

Primer	Sequence	Tm
Pmarn-Calm	F5'- TCA CAA CCT CCC TTC GAT TC -3' R5'- TCT CTT CCT CGG AGT CGG TC -5'	54.4 °C 56.6 °C
Actin-ex	F5'- CAA CGC TCC TGC CTT CTA TGT C -3' R5'- ATG ACG GAA GGC TGG AAG AG-3'	57.9 °C 56.9 °C
Pmarn- TOR 1	F5'- GCT TCT TAT GCG CCA ACC GAC TTT-3' R5'- TTT AGT GCC AGG CCT TCC CGA ATA-3'	60.2 °C 60.3 °C
Pmarn-TOR (OLD)	F5'- TTG ACG CTT TGC AAT CAC TC-3' R5'- TGG CCA GAA GCT TTT TCA GT-3'	53.9 °C 55.0 °C
Pmarn YAK1	F5'- GTG CGC GGC CTT CTA CAA ATC AAT-3' R5'- TGT TGC TGG ATA CCG GGA GCA ATA-3'	60.3 °C 60.3 °C

cDNA synthesis and initial primer testing:

The DNase treated RNA samples were used to synthesize cDNA using the BIO-RAD iScript Select cDNA synthesis kit. To each tube containing the 11µL of DNase treated RNA 4µL of 5x iScript select reaction mix, 2µL of Oligo$(dT)_{20}$ primers, 2µL of RNase-Free water and 1µL of iScript reverse transcriptase was added. For the initial primer testing, 50ng of the original isolated RNA (Diluted 1:100) of each sample (if the purity ratios were above 1.8) were added to 1µL of each forward and reverse primers (10µM) for YAK, Actin and two sets of TOR primers (TOR Old and TOR 1) along with 2µL GSP (gene specific primer) enhancer solution, water and 1µL of iScript reverse transcriptase. After gently mixing the tubes by tapping, they were placed in the BIO-RAD thermocycler at the following program:

Cycle 1: (1X)	Step 1:	42.0 °C for 60:00
	Step 2:	85.0 °C for 05:00
Cycle 2: (1X)	Step 1:	4.0 °C for ∞

Genomic Contamination check using Calmodulin primers

The 24 hours Mold/Yeast and Mold-Rapamycin/Yeast-Rapamycin cDNA samples were checked for any genomic contamination by conducting a PCR on the cDNA samples synthesized above using Calmodulin primers. For each sample, 1µL of cDNA was added to 10µL of GoTaq green master mix 2X from PROMEGA (Madison-Wisconsin), 2µL of the forward and reverse Calm F and Calm R primers and 5µL of RNase-Free water. A blank tube was also made with everything except the cDNA, instead 1µL RNase-Free water was added to make sure the reagents are contamination free as well. To compare, a reaction with 1µL genomic DNA isolated from Mold was also set up. The tubes were

then tapped gently to mix everything and placed in the BIO-RAD thermocycler at the following program:

Cycle 1: (1X)	Step 1:	95.0 °C for 03:00
Cycle 2: (34X)	Step 1:	95.0 °C for 00:30
	Step 2:	62.0 °C for 00:30
	Step 3:	72.0 °C for 01:00
Cycle 3: (1X)	Step 1:	72.0 °C for 07:00
Cycle 4: (1X)	Step 1:	4.0 °C for ∞

Each reaction product was run on a 2% Agarose gel to see any visible genomic contamination.

Primer check

For the initial primer testing, the PCR was conducted with primer sets of YAK1, Actin-ex, and two sets of TOR Primers (named as TOR OLD and TOR 1 primers). They were set up exactly the same way as above except 2 different concentrations (5pmol and 10pmol) to see which one worked better. The annealing temperatures were set ~2-4°C below the given Tm. A 2% Agarose gel was ran on the reaction products to see more efficient primer concentration.

Gradient PCR

The gradient PCR was set up to determine the closest annealing temperature of each of the primer sets in the range 51°C to 59°C. The PCR reaction mix was assembled in a similar fashion as above and set up in the BIO-RAD MJ Mini Thermal Cycler as follows:

Cycle 1: (1X)	Step 1:	95.0 °C for 05:00
Cycle 2: (34X)	Step 1:	95.0 °C for 01:00
	Step 2:	51 °C/59 °C for 00:30
	Step 3:	72.0 °C for 01:00
Cycle 3: (1X)	Step 1:	72.0 °C for 07:00
Cycle 4: (1X)	Step 1:	4.0 °C for ∞

The reaction products were then ran on a 2% Agarose to determine the most accurate annealing temperatures.

Quantitative (Real-Time) RT-PCR

To investigate the gene expression quantitatively, Qiagen Quanti-Fast SYBR green RT-PCR kit. The reactions were set up in a BIO-RAD 96 well clear PCR plate. Every reaction was set up in triplicate. Each reaction required the following recipe:

2x QuantiFast SYBR Green RT-PCR Master Mix (Buffer)	=	12.5µL
QuantiFast RT Mix (Enzyme)	=	0.25µL
RNase-Free Water	=	5.25µL
Primer F	=	2.50µL
Primer R	=	2.50µL
cDNA (regardless of the concentration)	=	2.00µL
TOTAL	=	25 .00µL

The first qRT-PCR was set up with just Calmodulin primers with cDNA sample of RNA collected from 48 hour Mold culture. This was so the standard curve could be determined and the best workable conditions of Calmodulin could be optimized. The master mix was

prepared for 35 reactions. Since, the Buffer is photosensitive, the tubes were covered in aluminum foil and exposure was avoided. In an eppendorf tube the calculated amount of Buffer, Enzyme, Water and Primers were added. On the BIO-RAD 96 well plate 2µL each of RNase-Free water was added to A1-3. To this, 23µL of the prepared master mix was added. This was the blank sample set up as negative control and to detect any contamination in the reagents. From B1-3 to H1-3 wells, 2µL of cDNA was added in the following dilutions, neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. These were the unknowns. The cDNA was quantified using a spectrophotometer and each sample was diluted to the concentrations of 2.5ng/µL, 0.25ng/µL and 0.025ng/µL for standards. 2µL of 2.5ng/µL was added to A4-6, this was 5ng of cDNA in each well. 2µL each of 0.25ng/µL and 0.025ng/µL were added to B4-6 and C4-6 wells respectively. 23µL of the master mix was added to each. Once the plate was ready, it was centrifuged for ~5-10minutes at 1,500RPM so that all the solution gets mixed and settled at the bottom. Place the plate in the BIO-RAD iQ5-Multicolor Real Time PCR detection system iCycler.

The program was set up on the BIO-RAD iQ5 software on the computer. The protocol SYBR green w.m.cSS (With melt Curve) was selected and a new plate created exactly the same way as the actual plate was loaded. The program was set up to conduct a standard curve in real time under the following thermocycler program:

Cycle 1: (1X)	Step 1:	55.0 °C for 10:00
	Step 2:	95.0 °C for 05:00
Cycle 2: (40X)	Step 1:	95.0 °C for 00:10
	Step 2:	56.5 °C for 00:30 *REAL-TIME*
Cycle 3: (81X)	Step 1:	55.0 °C for 01:00 *MELT CURVE*
Cycle 4: (1X)	Step 1:	4.0 °C for ∞

After the program ran, the data was analyzed. The standard curve displayed the efficiency of the reaction, the coefficient of the determination (R^2), the slope of the line and the y-intercept. The PCR product was ran on a 2% Agarose gel. The amplification curve, Melt peaks were analyzed to make sure that the reaction was clean and it completed without any errors. The concentration of cDNA that worked the best was determined comparing the gel product.

After the best cDNA was optimized, a second qRT-PCR was conducted with the 24 hour *Penicillium marneffei* samples of Mold/Yeast and Mold-Rapamycin/Yeast-Rapamycin. Master mix was created for Calm, TOR 1, Actin-ex, and YAK1 primers for 30 reactions each and the plate was set as follows:

Table 2. The 96 well plate setup for 24 hours samples.

	CALM			TOR			ACTIN			BLANKS		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	M1- 0.5ng			0.5ng			0.5ng			CALM		
B	M1- 0.05ng			0.05ng			0.05ng			TOR		
C	Y1-0.5ng			0.5ng			0.5ng			ACTIN		
D	Y1-0.05ng			0.05ng			0.05ng			YAK		
E	M1rap-0.5ng			0.5ng			0.5ng			YAK	M1	0.5ng
F	M1rap-0.05ng			0.05ng			0.05ng			YAK	M1	0.0.5ng
G	Y1rap-0.5ng			0.5ng			0.5ng			YAK	Y1	0.5ng
H	Y1rap0.05ng			0.05ng			0.05ng			YAK	Y1	0.05ng

After centrifuging the plate to mix all the reagents, it was placed in the thermocycler. The plate in the BIO-RAD iQ5 software was set up exactly as the table.

Amplification curve and melt curve peaks were obtained using the analysis tab once the program was completed. For each gene, the melting curves were determined by calculating the area under the curve by assigning a beginning and ending temperature to each peak. The data analysis was carried out by normalizing the gene expression to a reference gene, in this case to Calmodulin and Actin. Graphs were generated for relative fold expression and normalized expression using the software for both 0.25ng/μL and 0.025ng/μL dilutions of each condition.

RESULTS

Bioinformatics Analysis :

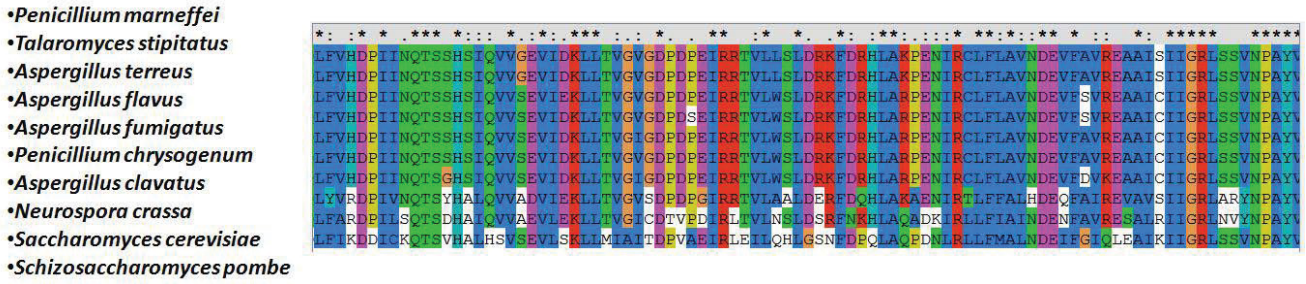


Figure 6. *Multiple Sequence alignment generated from the BLAST results using ClustalW2*

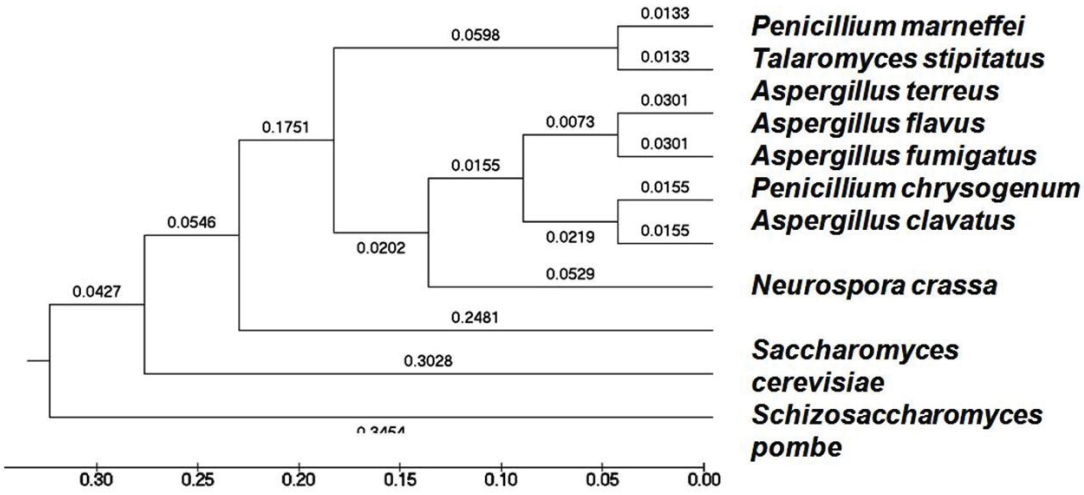


Figure 7. *Phylogenetic tree using MEGA4*

The TOR protein sequences in the NCBI databases were analyzed using various bioinformatics tools. BLAST results were filtered to show the top 10 fungal species most closely related to *Penicillium marneffei* to the TorA . The sequences were then aligned using Clustalw2 to conduct a Multiple Sequence Alignment (MSA). The MSA results showed the conserved domains which are the repeating sequences and structural domain

are represented by Figure 6. The strongly conserved regions are represented by **":"** and weakly conserved groups are represented by **"."**. **"*"** represents a singly fully conserved residue of the protein motif. The MSA data was used to generate a Phylogenetic Tree using Mega4. The phylogenetic tree represents the evolutionary relatedness of *Penicillium marneffei* to *Talaromyces stipitatus*. In contrast to fungal species where two homologs of TOR, TOR1 and TOR 2 are found, only one TOR ortholog has been identified in higher eukaryotes. Based on the BLASTp (protein BLAST) results, the serine/threonine-protein kinase mTOR in *Homo sapiens* bears 94% Query coverage to the TOR pathway phosphatidylinositol 3-kinase TorA in *Penicillium marneffei*(data not shown).

The protein structure analysis using SOPMA software reveals that it about 2382 amino acid polypeptide and weighs around 270kDa with the pI of ~6.40. About 65% of the structure is composed of alpha helices. Only ~2.6% composed of beta sheets and ~26% is composed of random coils and ~4.4%extended strands. Using PyMOL, it was observed that tertiary structure is stabilized by disulfide bonds, hydrophobic interactions, hydrogen bonds and ionic interactions.

Morphological Analysis using Phase Contrast Microscopy:

Images of the *P. marneffei* broth cultures grown in SAB were taken using an inverted phase contrast microscope at various magnifications to observe cell growth in normal conditions and in the presence of a growth inhibitor, rapamycin.

Figure 8. are the images from the un-treated *P. marneffei* cells grown in broth culture at 25 °C. The culture was grown upto five days and images were taken every 24 hours. The first set of sequential images from 1-5 were taken at 100X. "Day 1" was taken at 100X

and “Day 5” was taken at 40X to show denseness of the cultures. After 36-48 hours, the cells in the flask started to turn red in color.

Figure 9. are the images from of the un-treated *P. marneffei* cells grown in broth culture at 37 °C. The pictures were taken every 24 hours and the distinct unicellular yeast cells were visible from 24 hours onwards. The sequential images from 1-5 were taken at 100X. “Day 1” was taken at 100X and “Day 5” was taken at 40X to show denseness of the cultures. Unlike the mold cells yeast cultures remained the same whitish color. The cultures were not as dense and therefore more flasks of yeast were grown than the mold.

Figure 10. are the images from the Treated-treated *P. marneffei* cells grown in broth culture at 25 °C after 12 hours. The cultures were grown up to five days and images were taken at 12, 24 and 120hours. The first sets of sequential images were taken at 100X. “Day 1” and “Day 5” were taken at 40X to show denseness of the cultures. After 36-48 hours, the cells in the flask started to turn red in color. The growth was stunted and seemed far less than un-treated cells.

Figure 11. are the images from the Treated-treated *P. marneffei* cells grown in broth culture at 37 °C after 12 hours. The cultures were grown up to five days and images were taken at 12, 24 and 120hours. The first sets of sequential images were taken at 100X. “Day 1” and “Day 5” were taken at 40X to show denseness of the cultures. Unlike the mold cells, yeast cultures remained the same whitish color. The cultures were not as dense and therefore more flasks of yeast were grown than the mold. The growth was stunted and seemed far less than un-treated cells

1. Control (Un-treated) Mold and Yeast cultures at different time points

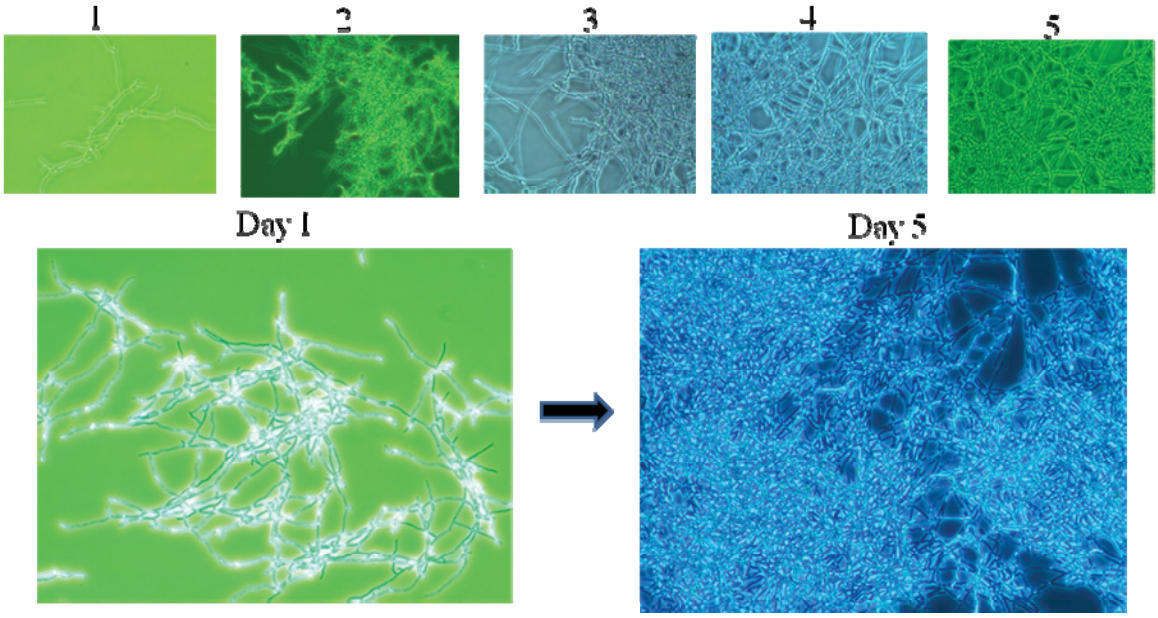


Figure 8. *Mold (25 °C) Day 1-5. The first set of sequential images from 1-5 were taken at 100X to show denseness of the cultures*

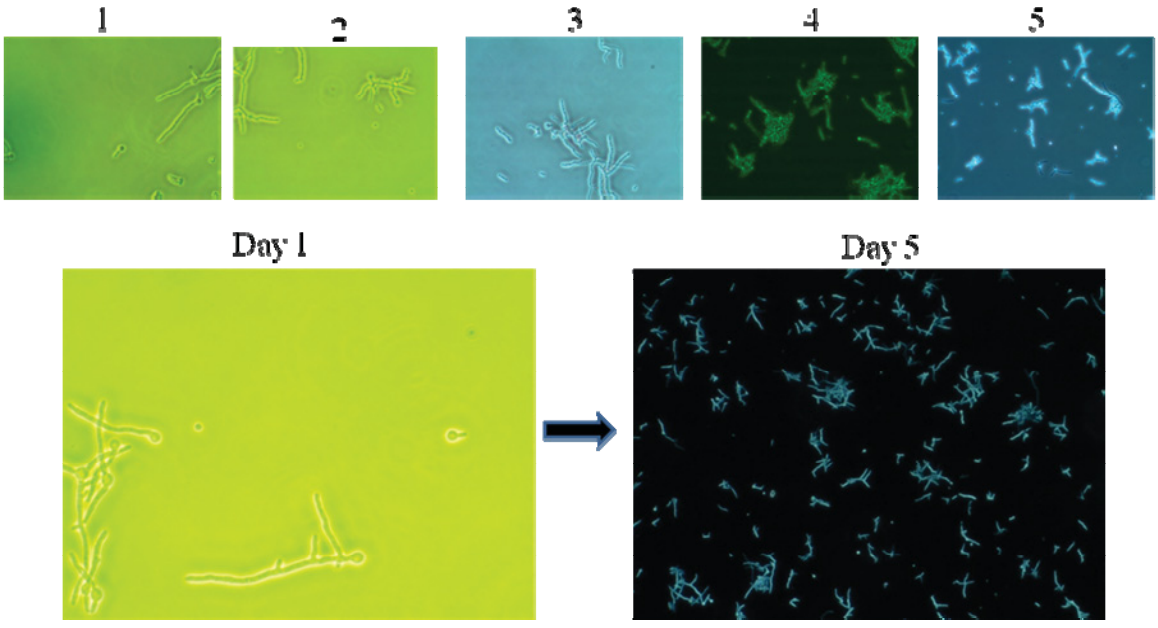


Figure 9. *YEAST (37 °C) Day 1-5. The first set of sequential images from 1-5 were taken at 100X. . The cultures were not as dense as the mold cultures*

2. Rapamycin treated cells

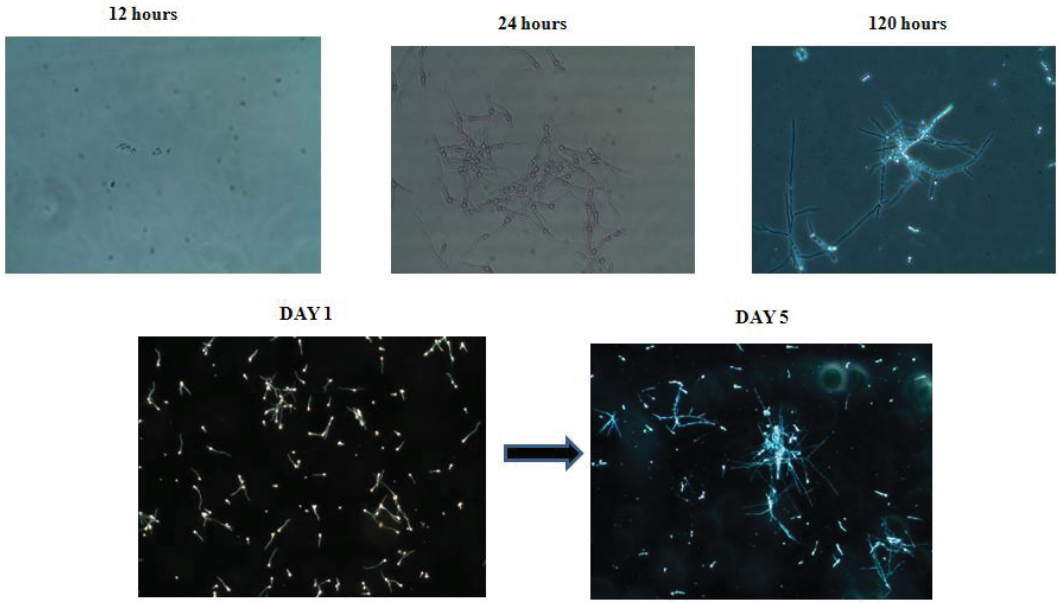


Figure 10. *Mold-Rapamycin(25 °C) 12 hours-120 hours. The first sets of sequential images were taken at 100X. The growth was stunted and seemed far less than un-treated cells*

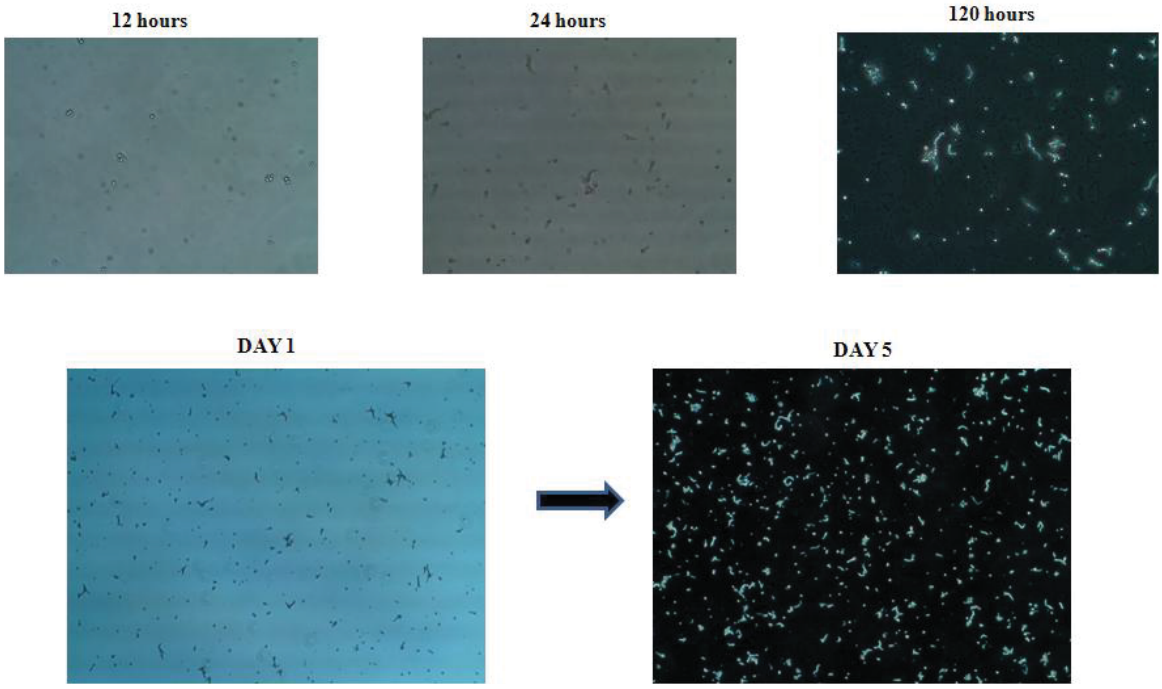


Figure 11. *Yeast-Rapamycin(37 °C) 12 hours-120 hours. The first sets of sequential images were taken at 100X. The growth was severely retarded and some conidia looked ungerminated.*

Cell Collection:

The concentrations of conidia harvested from the 10 day PDA plates varied anywhere between 20.0 x 10^7 to 90.5 x 10^7 conidia/mL. All the broth cultures were inoculated with a conidia concentration of 1 x 10^7 conidia/mL. The cells in the liquid broth cultures were collected at 24 hours and 120 hours. The weights of the pellets were ~500-800mg.

RNA and cDNA concentrations:

The selected RNA sample concentrations were as follows

Table 3. RNA quantification

SAMPLE	A_{260}	A_{280}	A_{260}/A_{280}	CONCENTRATION
M1	1.277	0.735	1.7386	510.949 µg/mL
Y1	2.534	1.363	1.8587	1013.428 µg/mL
M1rap	3.215	1.775	1.8110	1285.815 µg/mL
Y1rap	3.233	1.803	1.7927	1293.106 µg/mL

The concentrations of the cDNA synthesized from the above samples were:

Table 4. cDNA quantification

SAMPLE	A_{260}	A_{280}	A_{260}/A_{280}	CONCENTRATION
M1	0.460	0.251	1.8321	850.62 µg/mL
Y1	0.649	0.353	1.8414	1201.09 µg/mL
M1rap	0.916	0.480	1.9061	1693.76 µg/mL
Y1rap	0.826	0.427	1.9347	1528.53 µg/mL

Initial Primer Testing:

For the initial primer testing in Figure 12, 50ng of M1 RNA was used with 2 different concentrations of each primer set by adding 1µL(10pmoles) and 0.5µL(5pmoles) of each primer for each lane respectively. The 2% Agarose gel was ran at ~130 volts for 1 hour and 10minutes. For all the gels, ~15µL of sample was mixed with ~5µL of DNA dye loading buffer. For reference, the Amresco 100bp DNA molecular weight marker was used for all the gels. The Gel was stained in Ethidium bromide for 20 minutes and was viewed under UV lamp. The bands for each primer set Yak1, TOR(1), TOR 2(old) and Actin are visible for both the concentrations.

Gradient PCR:

Figure 13 represents the 2% agarose gel represents the gradient PCR conducted on the synthesized cDNA to determine the optimum temperature of each of the primers. This was critical for the qRT-PCR. The 100bp molecular marker from Amresco was used. The Yak1, Tor1, and Tor2 (old) primers were initially designed to synthesize the product of size no more than ~200bp. The Actin primers were designed so the product was around ~400bp. The primer concentration used here was 10µM At different temperatures provided in a gradient by the thermocycler, DNA bands can be seen for the primer sets Yak1 , Tor1, Tor2(old) at ~200bp at temperatures 54.2°C to 58.5°C and for Actin at ~400bp for temperatures 51.6°C to 56.0°C.

GELS of PCR products of TOR1, TOR 2(old), actin and Yak1 primers :

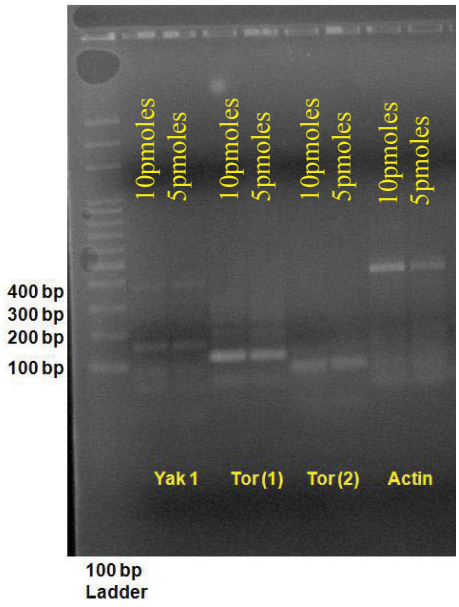


Figure 12. *Initial Primer Testing for different concentrations of TOR1, TOR2(old), actin and Yak1 primers*

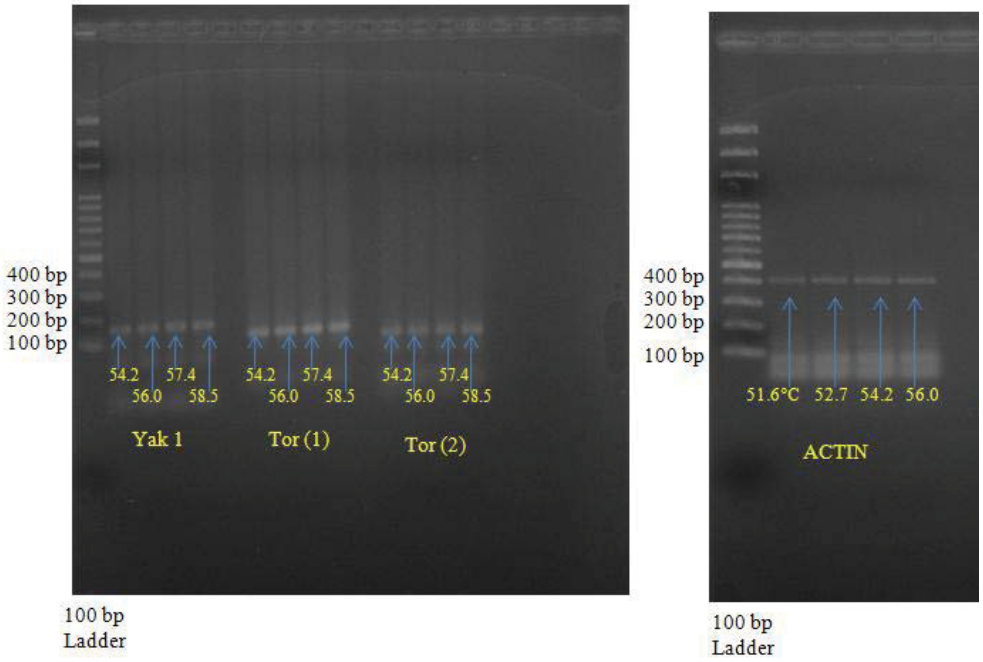


Figure 13. *Gradient PCR performed to determine accurate annealing temperatures of primers sets TOR1, TOR2(old), actin and Yak1 between the range 51 °C and 59 °C.*

GEL of PCR products of calmodulin primers

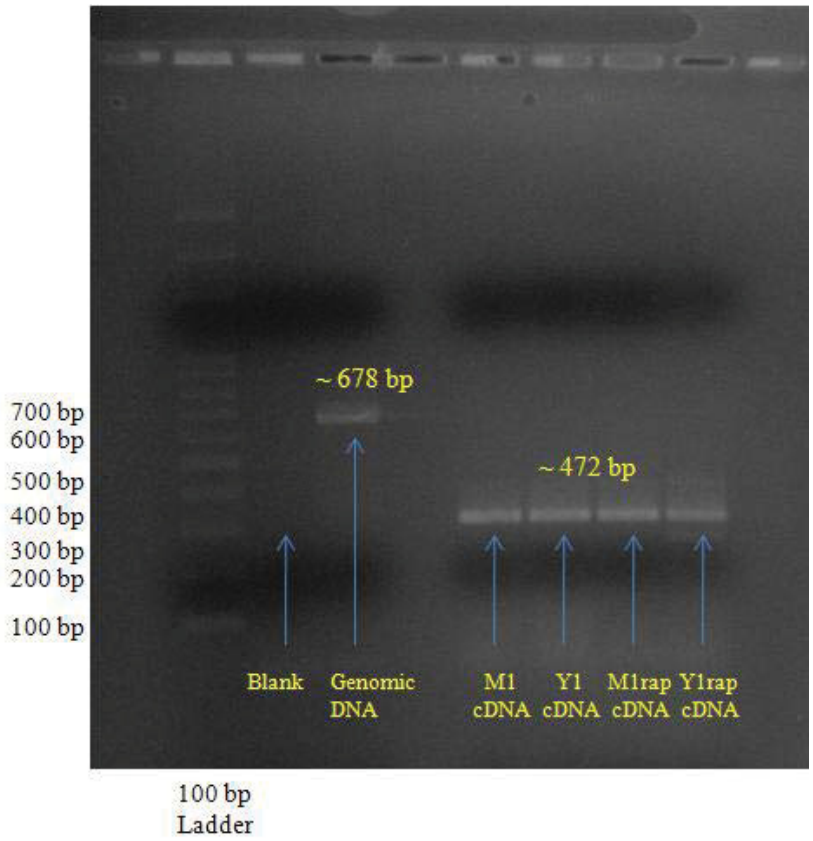


Figure 14. *PCR conducted with Calmodulin primers to detect any genomic contamination*

Genomic Contamination Check:

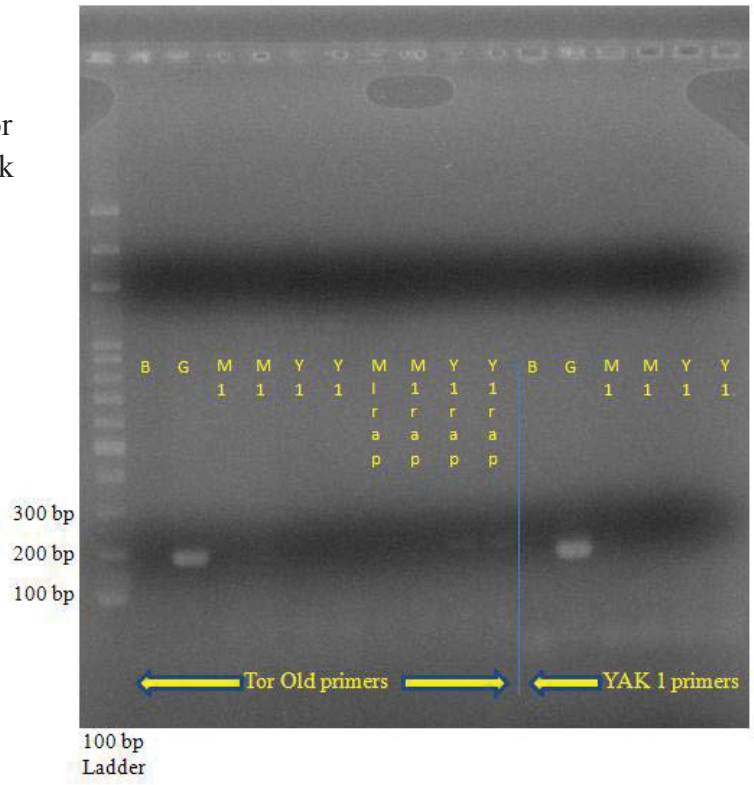
Figure 14. is the gel ran on the PCR products synthesized using the cDNA and Calmodulin primers. The optimum temperature of CALM F/R Primers for PCR has already been optimized to 62°C. This PCR was mainly run to see whether or not the cDNA samples are contaminated with the genomic DNA. A genomic DNA sample (180ng/µL) was run as a positive control, labeled as "G" on the gel. A blank sample with no template was run after the 100bp molecular marker from Amresco. Based on previously done experiments, the size of the genomic DNA and cDNA is around ~687 and ~472 respectively. The obtained gel results concurred with the prior findings. The

difference in the size indicated no contamination and therefore, the samples were used for qRT-PCR.

PCR conducted with other Gene Specific primer sets of TOR and YAK:

Figure 15 (a&b) were the gels ran the PCR products of the cDNA samples of M1/Y1 and M1rap/Y1rap. A genomic DNA sample and Blank sample was run with all the sets to make sure there is no contamination. The apart from the genomic DNA bands, very few bands are visible. The blanks were empty (as expected) for all the primer sets. The only visible bands were in the lanes where Y1rap was loaded. Since the expression is very low, it was necessary to run a qRT-PCR so that the expression can be quantified in real time. These gels were loaded with ~15μL of sample and ~5μL of Amresco EZ-vision, three dye loading buffer, 6X. These were directly viewed under UV light without staining with Ethidium Bromide.

a.) PCR using Tor 2(old) and Yak Primers.



b.) PCR using YAK and TOR-1

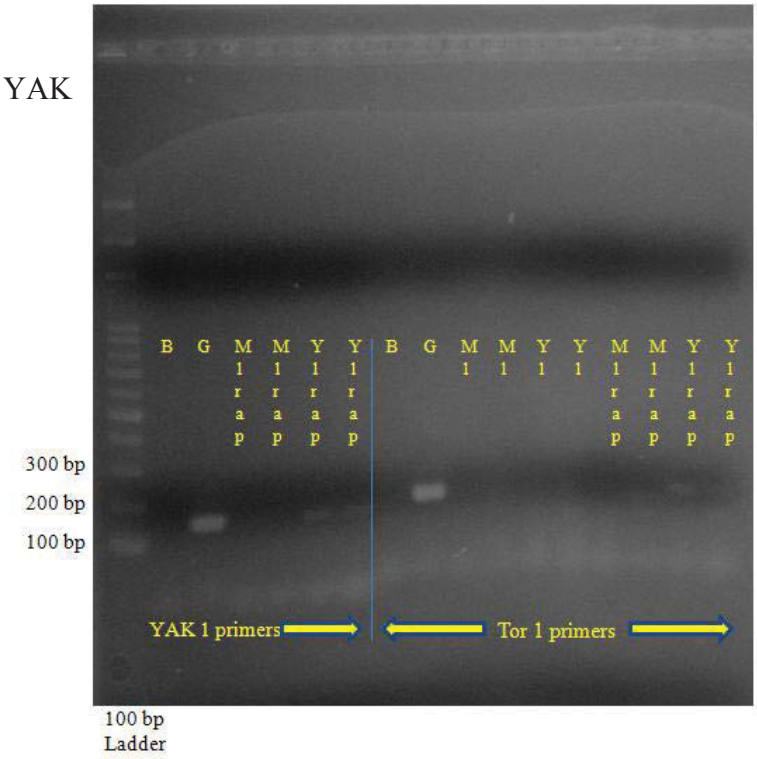


Figure 15 a. & b. *PCR conducted with different primers using cDNA samples of control and rapamycin treated cells to determine difference in gene expression using RT-PCR*

qRT-PCR with Calmodulin primers:

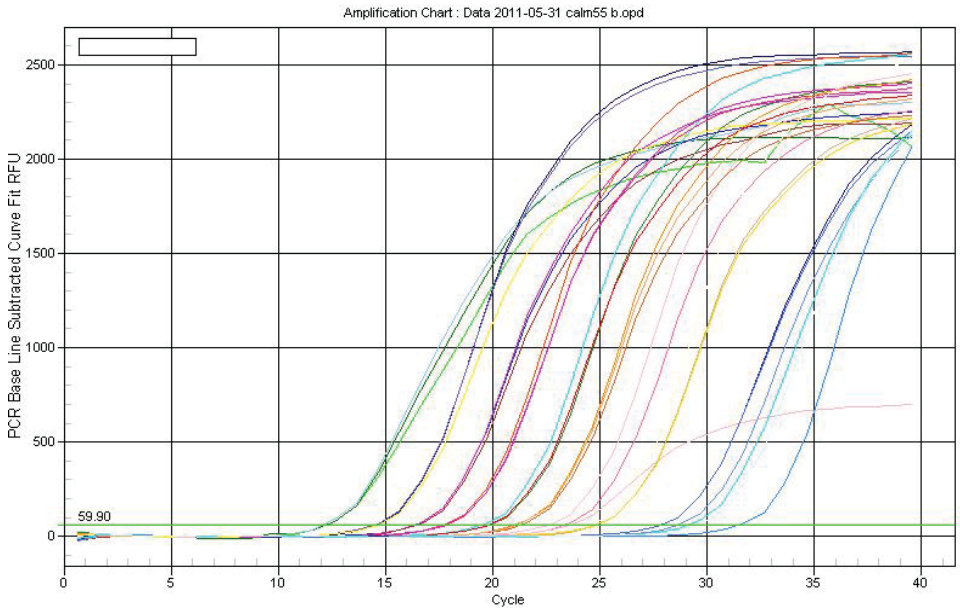
The first qRT-PCR was conducted using Calmodulin primers to optimize the conditions and figure out the best concentration of cDNA to work with. The cDNA used for synthesized using RNA collected from a 48 hour *P. marneffei* Mold liquid broth culture. The cDNA concentration was

Table 5. 48 hour Mold (un-treated) cDNA quantification

SAMPLE	**A_{260}**	**A_{280}**	**A_{260}/A_{280}**	**CONCENTRATION**
M2	0.459	0.248	1.8452	848.25 µg/mL

The reactions were set up using the SYBR Green I and the iQ^{TM}5 Optical System(BIO RAD). The BIO-RAD iQ5 software was used for analysis. The amplification chart in Figure 16(d) was analyzed to see if the cDNA template quantified or not. The melt curve peaks in 16(b) were analyzed to make sure all the peaks were at the same temperature of 56.5°C (optimized in earlier experiments). All the wells depicted a single peak and no contamination in the blanks or any other samples were detected. A gradient of concentration was run to determine the best workable template concentration from the neat cDNA to diluted cDNA to 10-6 as unknowns and known amounts 5ng, 0.5ng and 0.05ng as standards. Since each reaction requires 2µL of template which was obtained from 2.5ng/µL, 0.25ng/µL and 0.025ng/µL to make 5ng, 0.5ng and 0.05ng of cDNA template respectively in each set of reactions. All the reactions were run in triplicate. The standard curves were determined to evaluate reaction efficiency of 95.1% and an R^2 value of 0.992 which is close to 1 in Figure 16(c) is seen along with the plotted graph.

a.) Amplification curve



b.) Melt curve peaks

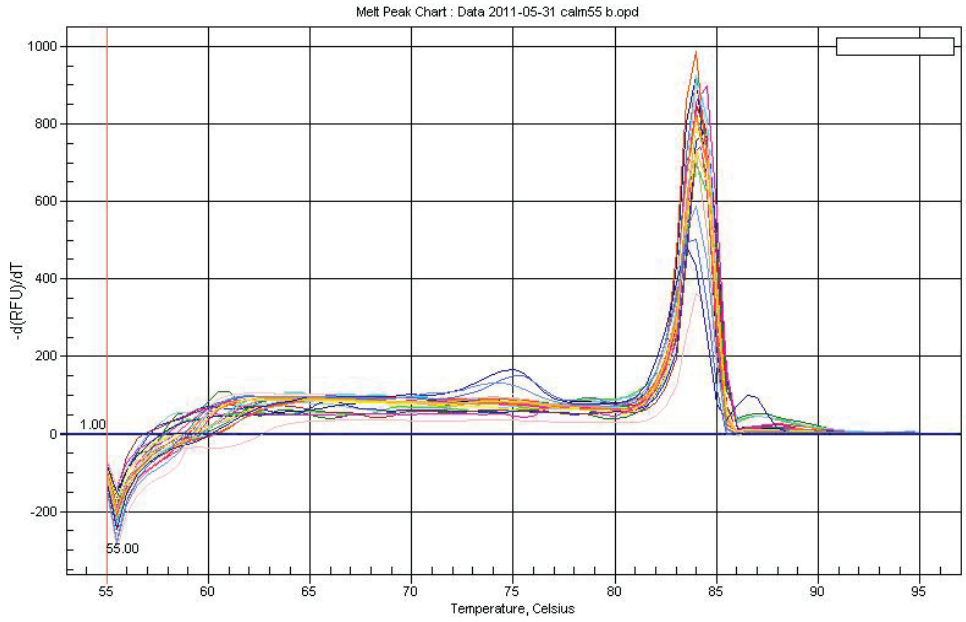
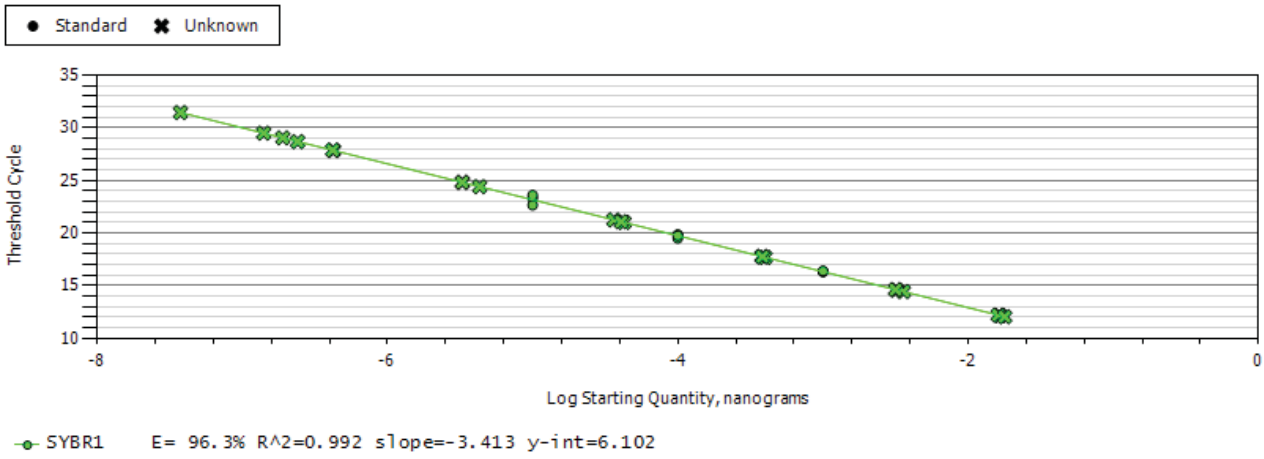
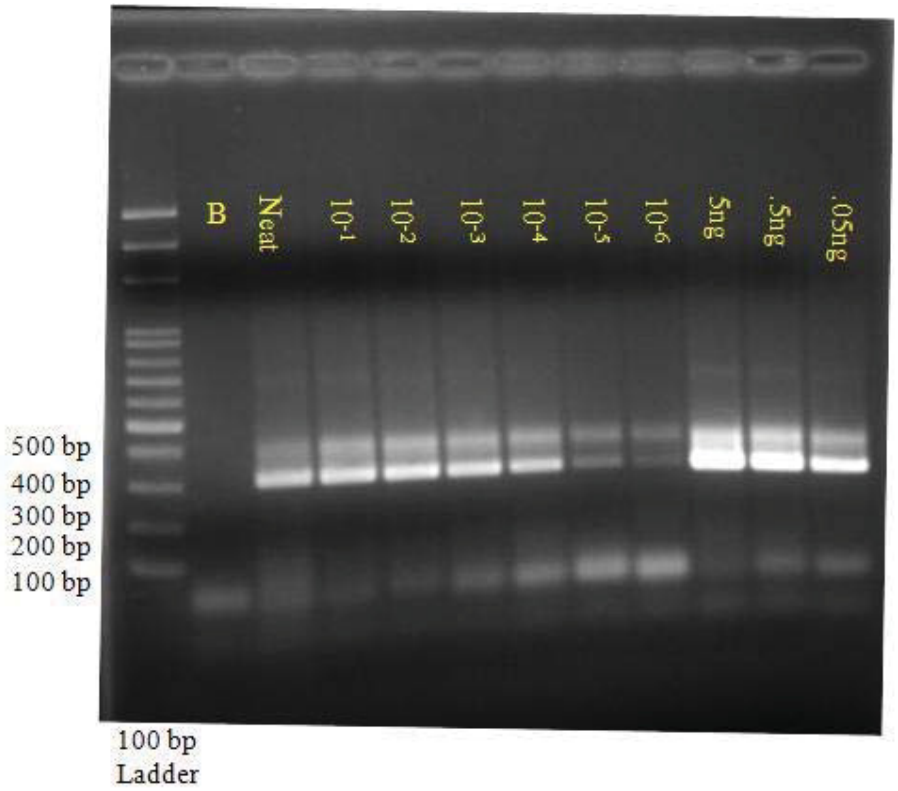


***Figure 16(a-b)**. qRT-PCR standard curve data. 16a.) represents the amplification chart showing the amount of DNA. 16b.) shows the melt curve peaks for quality control, all emerging at the same temperature*



c.) *Standard curve for Calm showing efficiency at 96.3% and R^2 at 0.992*



d.) *qRT-PCR product gel check of 48 hour Mold cDNA with Calmodulin primers*

Figure 16(c-d)16c. *shows the standard curve with the unknown dilutions. 16d. shows the qRT-PCR products on the 2%agarose gel.*

Figure 16(d) is the gel run on the qRT-PCR products. The blank lane was empty as expected and the lanes "Neat" to 10^{-3} showed bright bands and some smearing. Lane 10-4 to 10-6, the brightness of the bands decreased. The known sample of 5ng was the brightest and 0.05ng was the faintest.

Quantitative (Real-Time) RT-PCR with 24 hour samples:

Figure 17(a) describes the relative fold expression of Actin, Calmodulin and TOR for various samples at 0.25ng/µL (0.5ng) concentration (diluted 1:10 from the 2.5ng/µL). The general trend seems to be increase in expression for Yeast for both un-treated and treated cells. However, the relative expression levels after rapamycin induction increased almost 4 fold for Mold and about double for yeast. The relative expression of both Actin and Calmodulin was almost the same for the treated Mold.

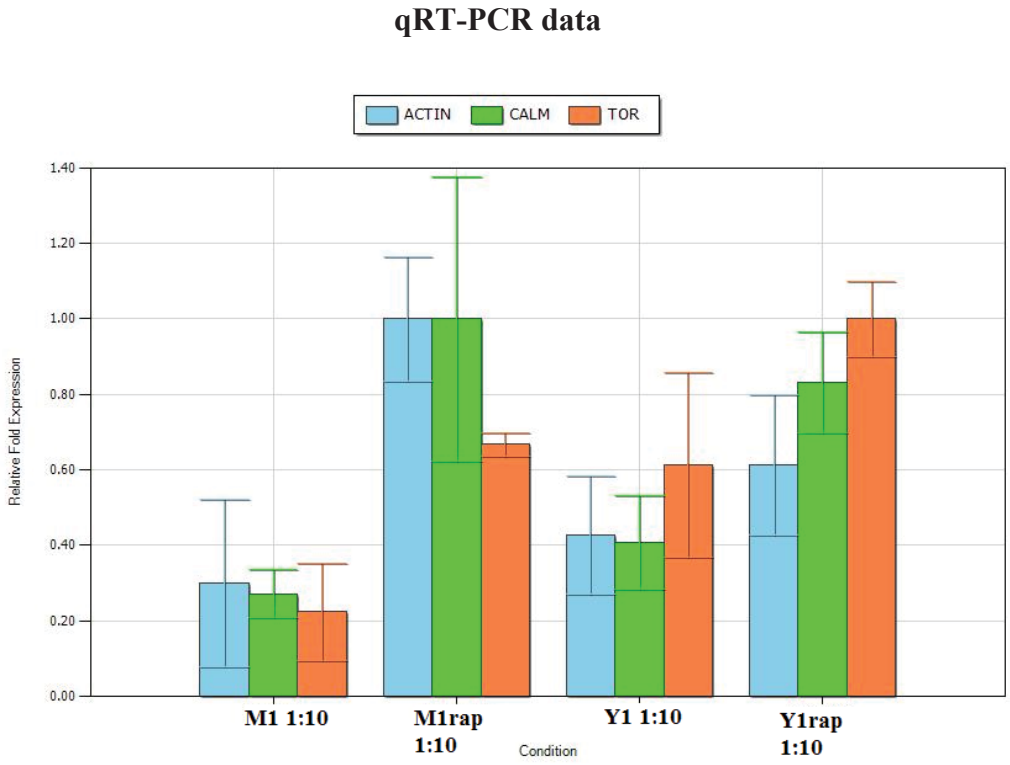


Figure 17. *(a) qRT-PCR data for 24 hours samples. Relative fold expression of the 0.5ng samples. The expression increases with rapamycin treatment*

Normalized fold expression was graphed for various conditions and genes. Figure 17(b) describes the Normalized expression of un-treated 0.5ng samples (0.25ng/μL) Actin. Calmodulin expression is almost the same in the both the conditions. Expression of TOR in Mold is half as in the Yeast samples. Yak expression increases significantly from Mold to Yeast. Figure 17(c) graphs Normalization of the un-treated 0.5ng samples (0.25ng/μL) to Calmodulin. Actin expression in both cases was almost similar (Mold was very slightly high). As for TOR and Yak, the expression in Yeast was significantly higher (almost double) than Mold. Figure 17(d) shows normalized expression of Calmodulin and TOR 0.5ng samples (0.25ng/μL) for all the conditions to Actin. Calmodulin expression remained almost similar except for the rapamycin treated Yeast sample. The expression of TOR was almost the same for un-treated and treated Mold cells. The yeast samples however showed significant increase in expression by increasing more than double. Then treated Rapamycin samples showed the highest expression. When the same samples were normalized to observe Actin and TOR expression normalized to Calmodulin in Figure 17(e), the expression of Actin remained almost the same except in the rapamycin treated yeast samples, where unlike Calmodulin, it decreased. Figure 17(f) and 17(g) show only the expression of TOR normalized to Actin and Calmodulin respectively for all the conditions. It is observed that expression increases in the yeast condition regardless of rapamycin induction. However, when normalized to Actin, rapamycin treated yeast samples show highest level of expression unlike when normalized to Calmodulin. TOR expression of rapamycin treated yeast cells is lower than the un-treated yeast cells. It is important to note that this difference with Actin and Calmodulin was why two controls were optimized.

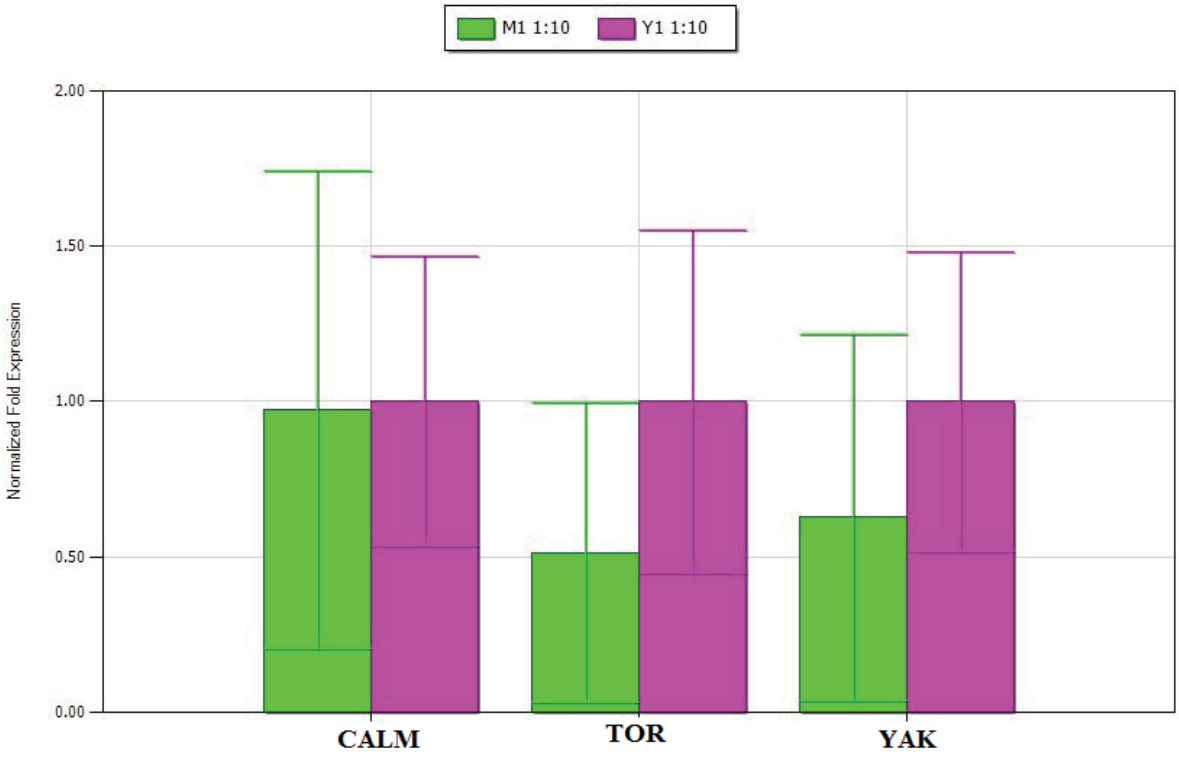


***Figure 17(b)**. Normalized expression of calmodulin, TOR and YAK to Actin. Calmodulim expression remains consistent in both mold and yeast phases*

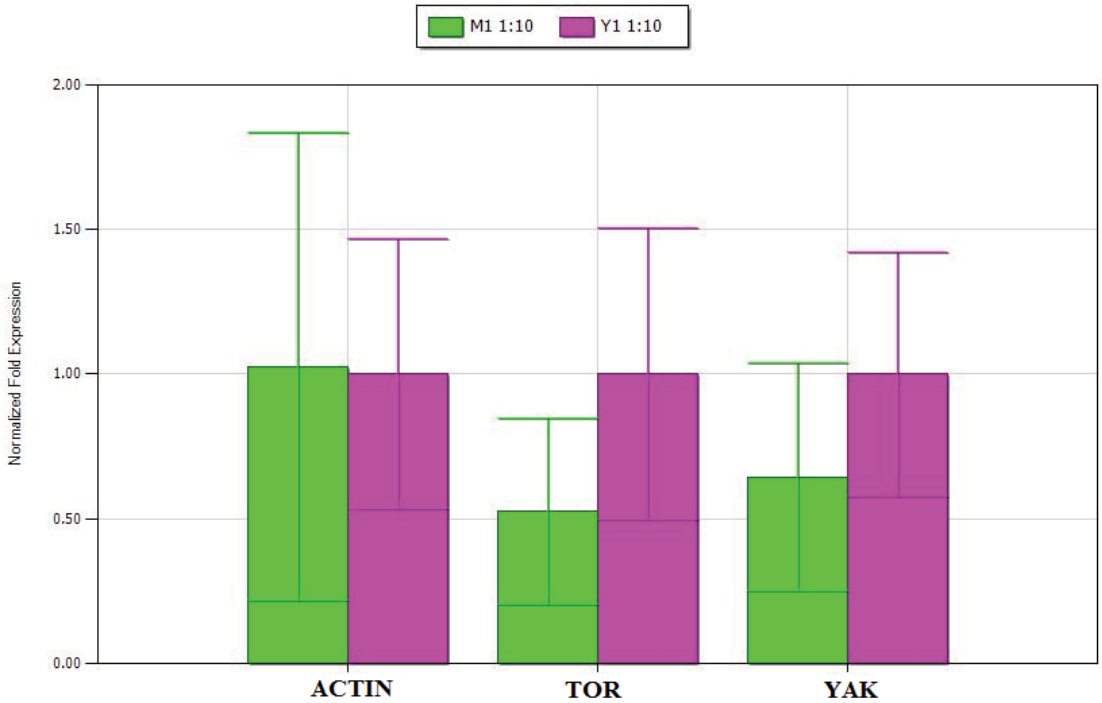


***Figure 17(c).** Normalized expression of actin, TOR and YAK to CALM. Actin expression remains consistent in both mold and yeast phases*

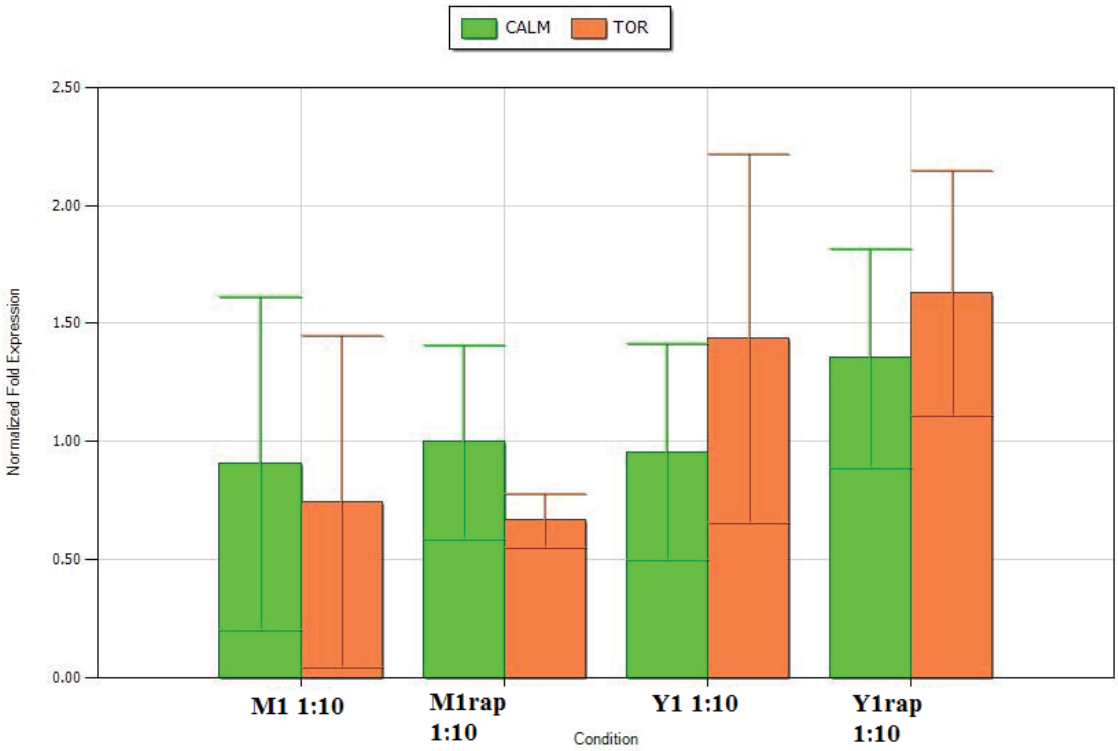


***Figure 17(d)**. Normalized expression of CALM and TOR to Actin for all conditions. CALM expression increases in yeast-rapamycin*

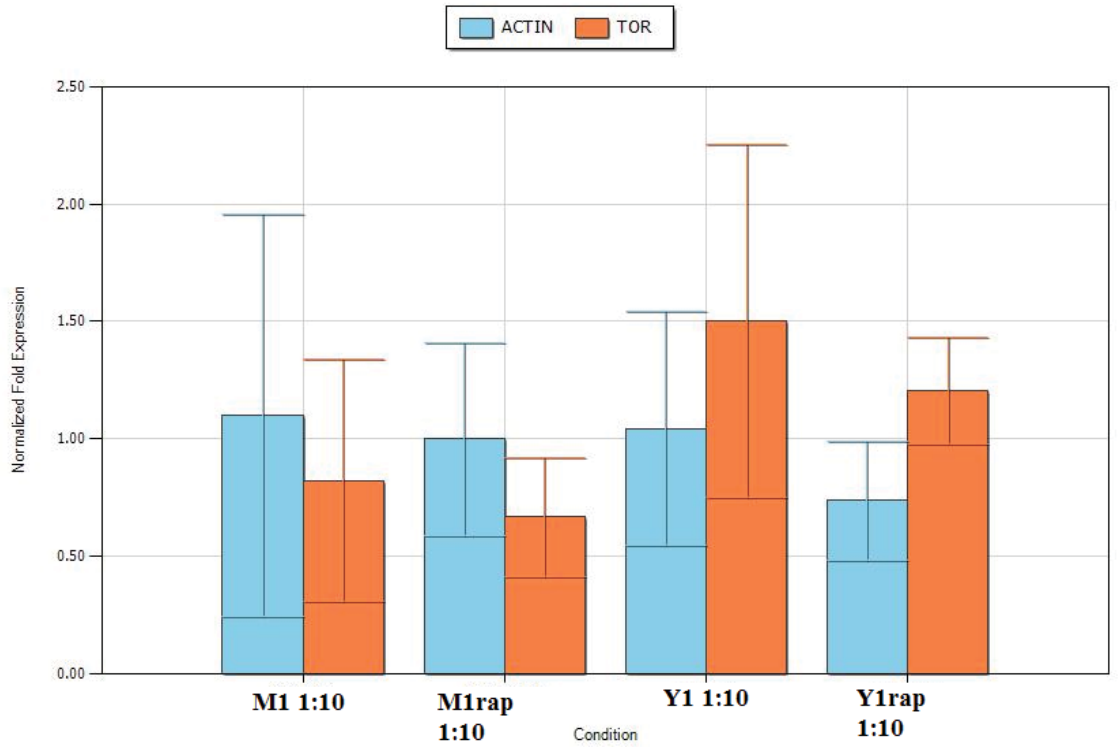


***Figure 17(e).**Normalized expression of Actin and TOR to CALM for all conditions. Actin expression slightly decreases in yeast-rapamycin*

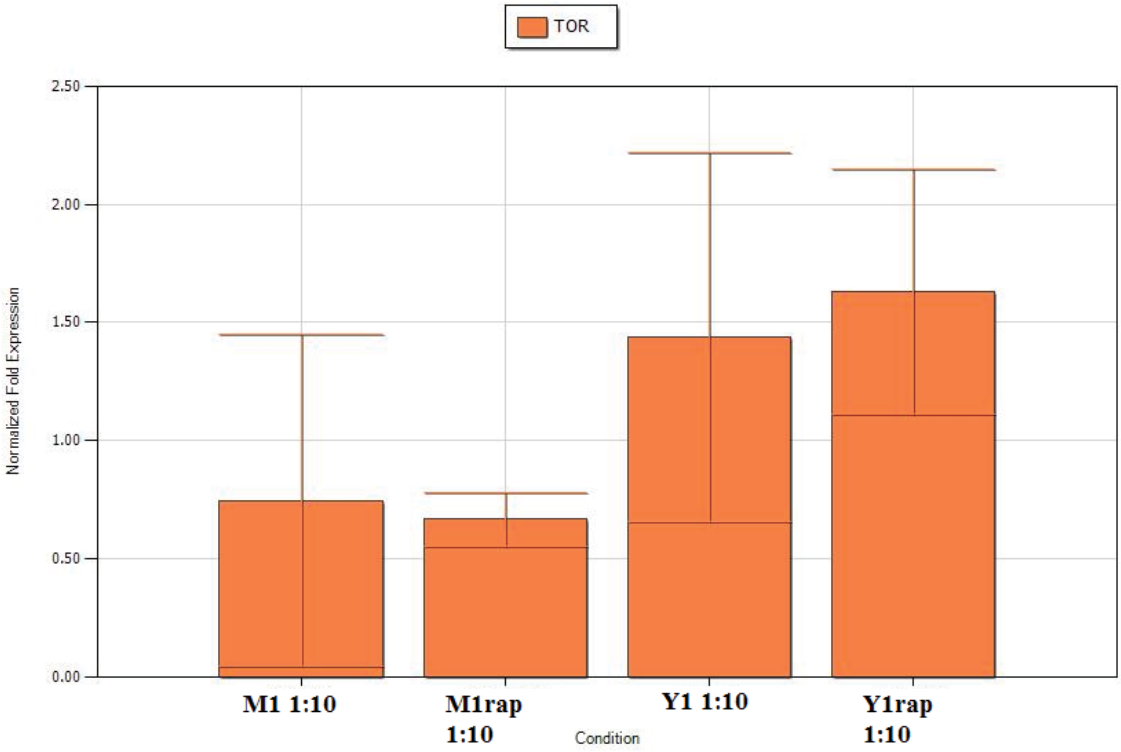


Figure 17(f). *Normalized Expression of TOR to Actin. The expression in yeast is 2-fold greater than mold. Expression in yeast rapamycin increases.*

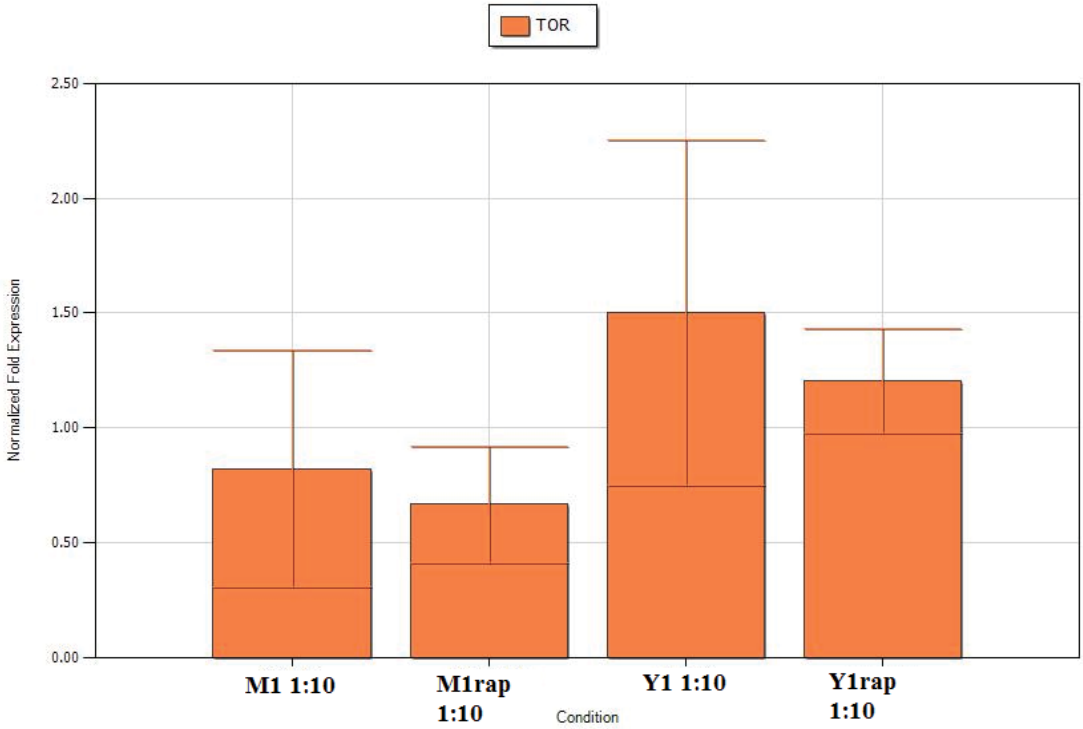


Figure 17(g). *Normalized expression of TOR to CALM. Expression increases 2 fold in control yeast and slightly decreases with rapamycin treatment.*

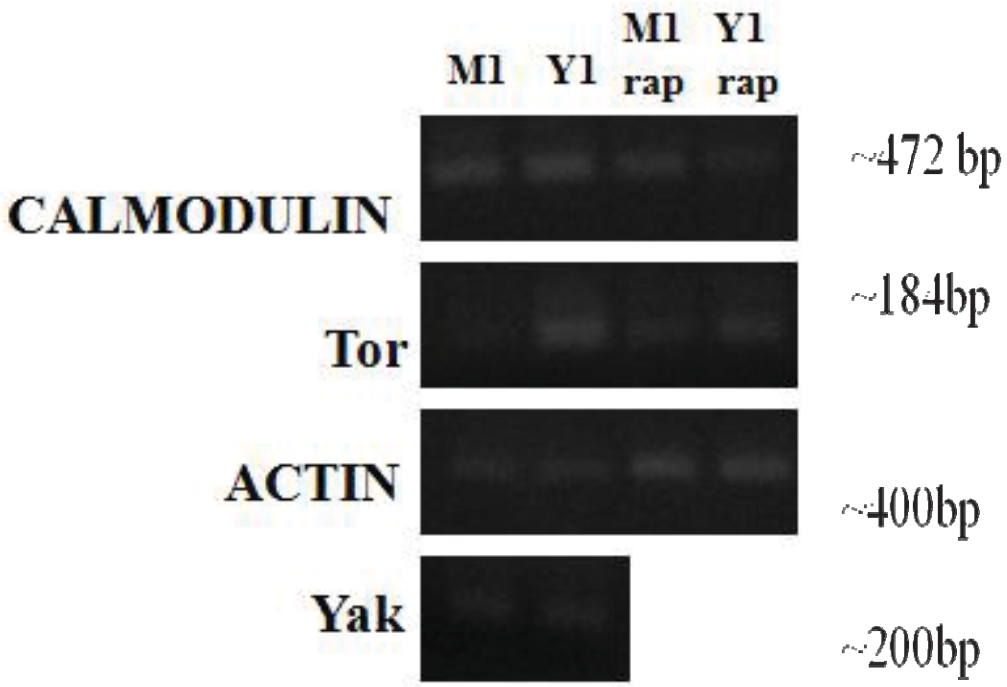


Figure 18. *Gel run on qRT-PCR products for 24 hours samples for calmodulin, TOR, actin and YAK to observe expression in different conditions.*

A gel was run on the qRT-PCR products for the 24 hour samples. All the samples chosen for the gel were 0.5ng (0.25ng/µL) diluted 1:10 from 5ng (2.5ng/µL). The size of the bands corresponded to the previous RT-PCR results. This gel simply shows the difference in expression as observed in the graphs in Figure 17. The most noted features of this gel are the expression of TOR that increases in the Yeast phase for both un-treated and treated samples. It is to be noted that the un-treated yeast expression is highest, showing the brightest band. However, the treated yeast sample is still brighter than the treated Mold sample, which concurs with qRT-PCR graph analysis. Actin expression increases with rapamycin induction but rapamycin treated yeast band is least bright. As for Calmodulin the difference is hard to point on the gel unlike the graphs for all the conditions. Yak analysis was only done on the un-treated cells and the increase in the yeast phase as shown in the graphs is visible on the gel.

DISCUSSION

Currently, the treatments for penicilliosis include use of various antifungal drugs such as Amphotericin B, itraconazole or voriconazole. All these drugs however, have only moderate effects and there is a need to find more effective and specific drug targets. Use of antifungal drugs has been most popular but it is often observed these drugs are more toxic to the patients and significantly decrease their life expectancy. Little work has been done with gene therapy to treat this disease.

The key to uncover effective treatments for penicilliosis is to unlock the mystery of the dimorphic switch in this pathogenic fungus. Since, TOR is one of the key regulators of cell growth and proliferation, it is critical to determine if it undergoes any significant changes during the dimorphic shift to the pathogenic yeast form.

In other fungal infections like candidoses, rapamycin has proved to be as effective as Amphotericin B. The reason it has not been followed to be a promising anti-fungal agent is its potent immunesupressive activity and its effect on the mammalian TOR in the patient. It is however important to explore Tor's role in dimorphism. It is the Tor's homology to various PI kinases which makes it a pivotal target for Rapamycin. Rapamycin binds to the intracellular binding proteins FKBPs. The FKBP-Rapamycin complex binds to TOR and blocks its function. Along with obstructing protein translation, this significantly hinders the signaling pathways of various kinases. It causes cell cycle arrest in G1 phase by drastically reducing the function of Cdk4/cyclin D and cdk2/cyclin E (Sehgal, 2003).

Interestingly, the effects of rapamycin as an antifungal agent are limited. Various TOR complexes react differently to rapamycin induction. The complex TORC2 that

mainly functions towards the polarization of actin cytockeleton, is insensitive to rapamycin induction. However, the functions of TORC1 complex are inhibited by-FKBP complex such as protein translation and repression of genes activated by environmental stress such as nutrient starvation. Therefore, Rapamycin activates the stress response of the cells, which is reflected by the trend towards higher expression of TOR in rapamycin treated Yeast cells. The data is not statistically significant as the differences are less than 0.5 fold in certain conditions and error bars are present.

It is also remarkable to note that the presence of rapamycin does not severely affect the actin cytoskeleton reorganization, as demonstrated in the morphological analysis of all the samples. Even after inducing rapamycin, the Mold cells gradually switch to produce some yeast cells at 37 °C successfully. However, the growth was severely retarded and a lot of ungerminated conidia were present. According to Crespo & Hall (2002), Inactivation of TOR by rapamycin induces autophagy even in rich nutrient environments. Despite this, the increased expression of TOR in the yeast cells could be attributed to two factors. Firstly, the yeast cells are larger in size and volume than individual mold cells and secondly, certain TOR interacting proteins like Avo1p and Avo3p are conserved in both fission yeast and mold (Inoki *et al*. 2005).

Consequently, due to the vast spread functions of TOR, it is imperative to explore the possibility of using more than one control (reference) gene. Previously conducted qRT-PCR studies show that actin is a reliable housekeeping gene for *P. marneffei.* However, it behaves erratically at times by giving multiple peaks, which could be explained by having more than one transcription sites. Also, from analysis of RT-PCR gels, it is evident that due to the specificity of the designed primers, there are no introns

present the amplified product. The size of the PCR product of the genomic and cDNA sample for actin was identical, which made it difficult to detect any genomic contamination. This called for an alternative control and calmodulin was thus chosen. The size difference in the genomic and cDNA PCR product was significant and clearly indicated if the extracted RNA sample was contaminated with genomic DNA. TOR and YAK did not have any introns either which was indicated in the same size of genomic PCR and cDNA PCR products. However, for qRT-PCR, both actin and calmodulin were used. Both are reliable housekeeping genes and are expressed equally in both the mold and yeast phases of *P. marneffei* regardless of time and culture condition. The qRT-PCR conducted on just Calmodulin was to determine the best working concentrations of the cDNA template and to optimize the thermocycler program. Hence, the concentration of cDNA template used for data analysis was the 1:10 dilution of 2.5ng/µL sample.

TOR functions critically at both transcription and translation levels. Increase in transcription does not indicate increase in translation. This can be studied using the phosphoprotein profiling of the *P. marneffei* cells. TOR regulates a serine/threonine phosphatase, PP2A (highly conserved in both yeasts and humans). PP2A functions to serve various cellular processes like DNA replication, transcription, translation, metabolism, proliferation, and cell transformation. Tap42p is a PP2A-interacting protein in Yeast (Inoki *et al*. 2005). Various studies done in *Saccharomyces cerevisiae* confirm TOR as an essential regulator of PP2A. TOR phosphorylates Tap42p both *in vivo* and *in vitro* in a rapamycin sensitive manner (Inoki *et al*. 2005). The protein profiles of un-treated P. marneffei mold and yeast samples indicate the production of certain phophoproteins only in the Yeast form. On the other hand, once the cells are treated with

rapamycin, the expression of these specific yeast phosphoproteins is severely hampered. Which opposes the qRT-PCR data as the expression of TOR is amplified indicating the presence of a mechanism by which the cells try to compensate for the growth retardation caused by rapamycin.

In conclusion, accepting the first hypothesis, there is a distinct difference in the expression of TOR in the mold and yeast phases of *Penicillium marneffei*. The expression of TOR increases in the Yeast phase, thereby, making it a potential drug target.

The second hypothesis is rejected as once the cells are treated with Rapamycin, the cells amplify the expression of TOR in order to survive. This indicates the presence of a compensatory mechanism by the TOR complexes not affected by rapamycin. To address the final hypothesis, from the morphological results, it can be noted that the growth after rapamycin induction is severely stunted, if not completely obsolete. It is also noteworthy, that rapamycin treated cells at 37 °C looked more filamentous and thereby indicating a critical role of TOR in bringing about the dimorphic switch.

Future work needs to be done to further investigate the specific TOR complexes involved in switching to the yeast form and targeting them exclusively to prevent the dimorphic switching by sequencing the qRT-PCR products. Bioinformatics analysis needs to be conducted on the sequence data to explore the different orthologs of TOR to design more proficient treatment strategies. It is also crucial to do an in depth study of the cell cycle genes, like CDKs and Cyclins and determine their role in bringing about the dimorphic switch in conjunction with TOR. Arresting the cells in G1 by targeting only the limited and specific genes could revolutionize the treatment of Penicilliosis by causing minimum damage to the host body and eradicating the pathogen completely.

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