EXPRESSION PROFILE OF *flbD* DURING MORPHOGENESIS IN THE DIMORPHIC FUNGUS *PENICILLIUM MARNEFFEI*

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Expression Profile of flbD during morphogenesis
in the dimorphic fungus *Penicillium marneffei*

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Abstract

Penicillium marneffei is a pathogenic fungus that is thermally dimorphic in nature. It is able to exist as mold at 25°C and as yeast at 37°C. With the emergence of the AIDS epidemic, the number of infections due to this fungus also grew. Penicilliosis due to *P. marneffei* is considered to be an 'AIDS defining illness.

The genes that may regulate the processes of conidiation and dimorphism in *P. marneffei* need to be examined in detail. One such gene is *flbD*, belonging to the family of *flb* genes. *flbD* is thought to regulate conidiation in *P. marneffei*.. This particular study attempts to characterize a mutant (I242) generated by *Agrobacterium*-mediated transformation (AMT). This mutant is able to form conidia at 37°C, compared to the wild type which grows as a yeast. We hypothesize that *flbD* over expression is in this mutant is responsible for this aberrant phenotype.

RNA was extracted from the wild type F4 strain of *P. marneffei* and the mutant I242 at 24 hours and 120 hours of growth (mold and yeast phases) in broth cultures. RNA from the conidia of both the strains was also collected. cDNA was synthesized from these samples. RT-PCR was conducted on the cDNA samples in an attempt to determine the expression profile of *flbD* in the wild type and mutant. The results show that, under conditions that do not favor conidiogenesis (growth in broth), *flbD* is expressed by strain I242, but not the wild type. This observation supports our hypothesis.

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Introduction

Penicillium marneffei is a dimorphic fungus belonging to the genus *Penicillium.* This fungus was first discovered at the Pasteur Institute located in Vietnam in 1959 (Segretain, 1959). The first case of penicillioisis due to *P. marneffei* was not documented until 1973 (DiSalvo, 1973).

This particular *Penicillium* species is the only one in the genus to exhibit dimorphism, i.e., dimorphism is the ability of the fungus to switch between two different growth forms. In *P. marneffei*, this dimorphic switch is dependent upon the temperature of incubation (Canovas and Andrianopoulos, 2007). Interest in *P. marneffei,* has gained over the last two decades (Cooper et al., 2000; Cooper et al., 2008). This is due to the increase in cases of infections in humans from this particular fungus. With the AIDS epidemic emerging as a significant threat in the late 1970s, the number of individuals with suppressed immunity also increased drastically**.** As a result, the number individuals susceptible to contracting infections due to *P. marneffei* have also increased (Cooper et al., 2008). Penicilliosis due to *P. marneffei* is now considered to be an AIDS defining illness (Cooper et al. 2008; Adiranopoulos, 2002; Li et al., 1992). In northern Thailand alone, over 6000 cases of penicilliosis due to *P. marneffei* in individuals with HIV and individuals with suppressed immunity were documented (Cooper et al. 2008).

The "natural reservoirs" of the fungus are four species of bamboo rats- *Rhizomys sumatrensis, Rhizomys pruinosus, Rhizomys sinensis and Cannomys badius* (Ajello et al., 1995). Although not much is known in regards to the role bamboo rats' play, it has been suggested that the rats play a role in 'facilitation of the life cycle' of *P. marneffei*

(Vanittanakom et al., 2006). Recently, a study was carried out to determine whether or not dogs could be a reservoir for infection in Northern Thailand (Chaiwun et al . 2011). *Pencillium marneffei* is known to infect dogs. However, prior to this study this animal model had not been examined. The results from the study indicated that dogs could be a possible reservoir for *P. marneffei.* However, further studies need to be conducted (Chaiwun et al., 2011). Another recent study examined the genotypes of isolates of *P .marneffei* from humans and bamboo rats from various areas. The isolates of *P. marneffei* were collected from different sites scattered across the Guangxi Province in the People's Republic of China. The sites were spread out over a span of 500 kilometers. The study attempted to determine whether or not the bamboo rats and the humans were exposed to the same reservoir of infection (Cao et al., 2011). Another possibility examined by the researchers was whether or not the rats act as a 'vector for human infection'. The researchers were able to show that *P. marneffei* appears to be closely linked with bamboo rats. The results from the molecular genotyping of the isolates showed only slight differences in the allele frequencies in the populations of both humans and bamboo rats (Cao et al., 2011). However, this particular study was unable to confirm the bamboo rats as the source of infection.

Although, not much is known about the origin of *P. marneffei*, more information is now known about what causes the infections in humans. It is thought the infection occurs when the conidia, produced as a result of asexual development, are inhaled. In healthy individuals, *P. marneffei* is targeted by the body's immune response. It is marked by the T-cells and engulfed by macrophages (Cooper et al. 2008). However, in individuals whose immunity has been compromised, the fungus is able to survive and replicates as unicellular yeast cells within the macrophages (Cooper et al. 2008). The symptoms of infection in

individuals with AIDS include anemia, weight loss, lesions on the skin and hepatomegaly (Mootsikapoun and Srikultbutr, 2006). The disease is difficult to distinguish from other mycoses like histoplasmosis. To correctly identify penicilliosis due to *P .marneffei* laboratory identification is required (Andrianopoulos, 2002; Mootsikapun & Srikulbutr, 2006; Wu et al., 2008). Infections can be fatal if they are left untreated. Infections due to *P. marneffei* can be treated by amphotericin B and itraconazole or voriconazole (Cooper et al. 2008; Wu et al., 2008).

Conidiogenesis and Dimorphism

Asexual development in *Aspergillus nidulans* has been studied extensively and may be compared to development in *P. marneffei*. The asexual development may be divided into two stages which are vegetative growth and conidial development. The growth stage includes the germination of the conidia and also formation of the mycelium. After a certain period of time the hyphal growth stops in some of the cells. These cells begin asexual development which consists of making the conidia (conidiogenesis) and maturation of the conidia (Adams et al., 1998).

Conidiogenesis involves the initial formation of a conidiophore from "foot cells". These cells contain multiple nuclei. The conidiophore stalk extends from the foot cells to form a vesicle. The vesicles give way to form two types of cells, each of which contains a single nucleus. These cells are called the metulae and phialides (Figure 1). Following mitotic cell division of the phialides, chains of conidia can be seen on the top (Mims, et al., 1988; Yu et al., 2006; Ni and Yu, 2007).

Figure 1: Conidiation in *Aspergillus nidulans* (reproduced from, Ni and Yu, 2007, with permission)

As mentioned previously, *P. marneffei* exhibits dimorphism whereby the organism possesses the ability to switch between yeast and filamentous mold forms. This process is known as dimorphic switching. Many pathogenic dimorphic fungi have been found to exist predominately in one of the two forms when the infection manifests within a host. For this reason it is thought the dimorphism facilitates pathogenicity (Berman and Sudbery, 2002; Adrianopoulos 2002, Lengeler et al. 2000).

Penicillium marneffei, is thermally dimorphic. The life cycle of *P. marneffei* has been described by Adrianopolous (2002) and is divided into three stages: a vegetative hyphal growth at 25°C, asexual development (conidiation) at 25°C, and yeast growth at 37°C (Figure 2). This is similar to the asexual development of *A. nidulans.* At 25°C, during the saprotrophic growth phase, *P. marneffei* exists as septate, branched hyphal cells containing multiple nuclei. The hyphae produce conidia, which under certain growth conditions can

germinate to produce vegetative hyphal growth form. The presence of multiple nuclei indicated that nuclear and cellular divisions are not coupled. However, at 37°C nuclear and cellular division in *P. marneffei* are coupled and this results in the development of hyphae which are uni-nucleate and highly branched. At this temperature, a process known as arthroconidition is activated. The arthroconidia are released from the hyphae along the septal division planes. These continue to grow via fission to become yeast cells. These cells resemble the pathogenic yeast form found in human host tissue (Canovas and Andrianopoulos, 2007).

Figure 2: Representation of the life cycle of *P. marneffei* (reproduced from, Canovas and Andrianopolous, 2007, with permission).

Certain environmental triggers are able to initiate asexual development in *P. marneffei*. This development begins after hyphae growth is complete which is indicated by a multinucleate stalk growing away from the mycelium (Andrianopoulos, 2002). The regulatory pathway for asexual development has been studied extensively in *A. nidulans*. It has been proposed that the pathway of genes controlling conidiation is as follows: *brlA*→

abaA→ *wetA.* This gene cascade activates other genes resulting in the development and maturation of the conidia (Adams et al. 1998; Boylan *et al*., 1987; Roncal and Ugalde, 2003; Cánovas and Andrianopoulos, 2007). *brlA* activation is thought to be needed for development of conidia. The protein BrlA is found in vesicles, metulae and phialides. However, it is absent from the hyphae or mature conidia. This suggests the possibility that its primary function would be to regulate the development. This possibility was studied by Adams et al. (1988). Over expression of the gene *brlA,* prevented the growth and differentiation of the conidia (Adams et al., 1988; Adams and Timberlake, 1990).

The second gene in the pathway is *abaA* (abacus) gene. It was found to be activated during the intermediate stages of conidiation in *A. nidulans*. *abaA* mutants were found to form normal conidiophore structures. However, they were unable to produce chains of conidia as seen in the wild type. Instead, they produced abacus like structures at the ends of the conidiophore stalks (Ni and Yu, 2007; Sewall et al, 1990a; Adrianopoulos and Timberlake, 1994). This particular gene has been implicated in differentiation of phialides (Sewall et al., 1990a). The expression of *abaA* was found to be dependent upon the activity of *brlA (*Boylan et al, 1987).

The *wetA* gene was found to be needed during later phases of development for components of the cell wall (Sewall et al., 1990a). Furthermore, it was determined that *abaA* is needed in order for *wetA* to be expressed correctly. It was also determined that overexpression of *wetA* prevents growth of hyphae. It is thought that in *A. nidulans wetA* is responsible for activating genes that deal with formation and maturation of the spores (Marshall and Timberlake, 1991). *wetA* was also found to regulate its own expression and also that of the gene *brlA*. A homologue for *wetA* in *Penicillium chrysogenum* suggests that

systems for control of spore formation and development have been conserved in *Aspergillus* and *Penicillium* (Prade and Timberlake, 1994; Roncal and Ugalde, 2003).

Upstream Activators of Conidiation

The first gene in the cascade of genes involved in with the activation of *brlA* is *fluG.* In mutants of *A. nidulans* where there was a loss of function of *fluG*, conidiation was not observed nor was there any production of sterigmatocystin (Lee and Adams, 1994; Hicks et al., 1997; Yu and Keller, 2005). In mutants where FluG was overexpressed, activation of *brlA* resulted in formation of conidiophores in submerged cultures. This finding suggested that FluG may play a direct role in regulation of conidiation (Lee and Adams, 1996; Adams et al., 1998; D'Souza et al., 2001).

Furthermore, in *A. nidulans* it was discovered that conidiation may still occur if the *fluG* mutants are grown in proximity of the wild type. This indicated that the protein Flu G is involved in synthesizing an 'extracellular sporulation factor' (Adams et al., 1998). It was thus proposed that activity of the protein FluG resulted in two distinct functions. The first of which was the related to the development of conidia; this development required the activation of other *flb* genes (Adams et al., 1988; Wieser and Adams, 1995; Lee and Adams, 1996). The second function was activation of the protein FlbA which then inactivated FadA signaling (Yu et al., 1996). Both were found to be needed for development to occur.

The impact of FluG activity was studied by analyzing the suppressor mutations (suppressor of *fluG [sfg]).* These mutants are able to avoid the need for *fluG* in conidiation and sterigmatocystin production (Seo et al. 2003; Seo and Yu, 2006). It was proposed that the *fluG* dependent initiation of asexual sporulation is independent but parallel to G-

protein-mediated growth signaling (Yu et al., 1996, Rosen et al., 1999). The underlying principal for this particular hypothesis was that mutations in the proteins FluG or SfaD (suppressor of *FadA*) were unable to suppress the activity of *fluG* (Yu et al., 1996, Rosen et al., 1999).

Identification and characterization of the suppressor *SfgA* led to a modified model of conidiation in *A. nidulans* in which *SfgA* suppresses asexual development. The activity of FluG was needed to remove the intracellular repressive effects imposed by *SfgA* (Figure 3)

Figure 3: Modified model for conidiation in *Aspergillus nidulans* showing the action of the repressor *SfgA* on the *flb* family of genes (reproduced from, Mah and Yu, 2006, with permission).

The functions of the homologue of *fluG* in *Aspergiullus fumigatus* (*AffluG*) was studied by Mah and Yu (2006). The results from this study determined that in mutants with the *affluG* gene deleted, sporulation occurred normally on solid media. This suggested that FluG activity is not needed in the presence of air for sporulation to occur (in *A. fumigatus)*. However, it was determined that the mutants did not produce conidiophores in submerged liquid cultures, which the wild type strains of *A. fumigatus* were able to do (Mah and Yu, 2006).

Although an *AffluG* deletion mutation showed reduced conidiation, *AfbrlA* expression was found to be delayed in these mutants. From their studies, Mah and Yu (2006) were able to conclude that the presence of air may be able to compensate for the need for *AffluG* in developing the conidiophore structure. However, it still plays a role during *A. fumigatus* conidiation as well as in the expression of *afbrlA.* This also suggested that there is more than one pathway to activate the expression of *AfbrlA.*

The *flb* Family of Genes

The inactivation of genes *flbB, flbC, flbD* or *flbE* in mutants of *A. nidulans* resulted in delayed conidiation (Wieser et al., 1994). The mutants retained the ability to produce conidiophores in colonies over time (Weiser and Adams, 1995).

As mentioned previously, *abaA* is considered to be essential for the initiation of conidiation in *A. nidulans.* This model may also be applied to the *Penicillia.* Six genes that are thought to be important for the correct expression of *brlA* are *fluG, flbA, flbB, flbC, flbD and flbE.* Mutations in any of these genes results in conidia that appear to be fuzzy with cells that are undifferentiated (Adams et al., 1998). It was determined the order of these genes was *fluG*→*flbC*→ *flbE*→*flbD*→ *flbB* (Weiser and Adams, 1995; Lee and Adams, 1996). In *A. nidulans,* mutants in which *flbB, flbC, flbD or flbE* were inactivated delays in conidiation were observed (Wieser et al., 1994).

In mutants where the *flbA* loss of function occurs, the colonies begin as fluffy non-conidial colonies. However, by the third day of inoculation the colonies begin the process of disintegration and by the fifth day the colonies are found to be autolyzed (Lee and Adams, 1994b; Wieser et al., 1994). Overexpression of *flbA* led to the *brlA* activation and conidiation (Lee and Adams, 1994b). FlbA was found to be a regulator of G protein

signaling (RGS) and was needed for controlling vegetative growth signaling (Yu et al., 1996; Rosen et al., 1999; Seo et al., 2005; Seo and Yu, 2006).

The *flbD* gene is thought to encode for a 308 aa polypeptide (Weiser and Adams, 1995). Over expression of this gene in submerged cultures led to the formation of complete conidiophores with stalks, vesicles, sterigmata and viable conidia. This is different from mutants with overexpression of *brlA* or *flbA* which resulted in the formation of abnormal conidiophores (Adams et al., 1988; Lee and Adams, 1994b). Additionally, it was determined that the proteins FlbC and FlbD influence activation of development independently. Mutations in both *flbC* and *flbD* were found to have a combined effect on development (Weieser et al., 1995). Also, the over expression of *flbD* caused development in the absence of *flbC* (Wieser et al., 1995). Furthermore, *flbD* mRNA was found to be at a steady level in vegetative hyphae and also after development has been initiated in wild type cells. It was proposed that activity of the FlbD protein was regulated in response to sporulation signals and that these signals resulted in the activation of other regulators such as *brlA* (Weiser and Adams, 1995).

The gene *stuA,* which regulates *brlA* and *abaA,* was found to be needed for metulae and phialide formation during conidiation, but was not needed for the production of the conidia (Borneman et al., 2000). The figure below shows the proposed gene cascade for conidiation in *P. marneffei* (Figure 4).

Figure 4: Model for conidiation in *P. marneffei* (reproduced from, Canovas and Adrianopoulous, 2007, with permission).

Conidiation Mutant of *P. marneffei*

An *Agrobacterium*-mediated transformation (AMT) system was developed to analyze gene function in *P. marneffei* (Kummasook et al., 2010). The AMT system was employed to generate mutants with both known and unknown defects. The study and characterization of these mutants may increase the understanding of certain genes that are involved in the dimorphism of *P. marneffei* as well as its pathogenicity.

A mutant generated by the AMT system (A 23 242/ I242) maybe used to examine the effects of the *flbD* gene in *P. marneffei.* This particular mutant was identified as having a mutation in the promoter region of *flbD.* Curiously, strain I242 does not form the yeast phase when incubated at 37°C, as does the wild type strain of *P. marneffei*, but instead continues to produce conidiophores bearing chains of conidia (see below). By studying this mutant and comparing it to the wild type of *P. marneffei* more may be determined about the function of this particular gene and the role that it may play in conidiation.

Purpose of study:

The purpose of this particular study is the characterization of the AMT-generated strain I242 of *P. marneffei*. This particular mutant was identified as having a mutation in the promoter region of *flbD* (Kummasook, 2010). The phenotypic characterization of the mutant would include growth on several different media at both 25°C and 37°C. To study the morphology, the mutant will be examined by macroscopic, microscopic and molecular methods. The genotypic characterization of the strain I242 would be focused on determining expression levels of *flbD* during morphogenesis by using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) protocols.

Hypotheses:

1. *flbD* expression in the wild type and mutant strain I242 should be different. The difference will be more pronounced at 24 hours of growth as one would not expect conidiation to occur in the wild type after just 24 hours.
2. Over expression of *flbD* will result in increased conidiation.

General Research Protocol:

- Grow fresh cultures of F4 wild type *Penicillium marneffei* and *I242* at 25°C and 37°C on PDA.
- Collection of conidia at ten days of growth from both strains. Use conidia to inoculate Sabouraud dextrose broth (SAB) and incubate cultures at 25°C and

37°C. These cultures will be grown for 24 hours and 120 hours. The morphology of each of the samples would be examined.

- Cells are to be collected from these cultures (wild type and I242) for RNA extraction. Additionally, RNA is to be extracted directly from wild type and I242 conidia (25°C).
- Purification of RNA using DNase treatment, then use the RNA for complementary DNA (cDNA) synthesis.
- Adjustment of concentrations of samples to ensure that the differences in expression are not due to differences in concentration. Perform RT-PCR with Calmodulin primers to check for genomic contamination. Subsequently, perform RT-PCR with *flbD* start (5') and end (3') primers to determine differences in expression between the wild type and mutant.

Materials and Methods:

Chemicals, Reagents and Media:

All chemicals, reagents, and media (unless otherwise noted) were obtained from Bio-Rad Inc. (Hercules, CA), Amersco, Inc. (Solon, OH) or Fisher-Scientific (Pittsburgh, PA). The potato dextrose agar (PDA; Difico brand) and Sabouraud dextrose broth (SAB; Difico brand) which were used for culture media, were manufactured by Becton, Dickinson, and Co. (Sparks, MD). All media and solutions were prepared per the manufacturer's protocols using distilled-deionized water (ddH_2O).

Penicillium marneffei growth:

The wild type (WT; strain F4) of *P. marneffei* and strain I242 of *P. marneffei* (Kummasook, 2010) were both grown on PDA in 150 cm^2 cell culture flasks at both 25°C and 37°C. The wild type strain was taken from a stock strain from Chiang Mai University in Thailand and maintained at YSU. Both the mutant and wild type were grown for a period of 8-10 days.

Broth Inoculation:

To inoculate the broth, conidia were first collected. The conidia were collected from the PDA flasks. Approximately 10-15 ml of sterile ddH_2O was pipetted into the flasks and the plate was scraped gently using a cell scraper. The conidia suspension was then passed through a screen cap (Bio-Rad) containing a 1 inch glass wool (Corning Acton, MA) which was screwed on a 50ml conical centrifuge tube. The samples were then centrifuged for 30 seconds at 1000 RPM (150-200 g). The supernatant was discarded and the remaining

conidia suspended in a small amount of SAB. The final concentration of the conidia was determined using a hemocytometer.

Six separate 500 ml Erlenmeyer flasks containing 50 ml SAB were inoculated with concentrations 1 x 10^7 conidia per ml. The inoculated SAB broth was cultured in shaking water baths with temperatures set at 25°C and 37°C. The cultures were collected at 24 hours and also at 120 hours. Two of the flasks were of wild type strain and two flasks were of the *flbD* mutant cultures. A flask of each strain was kept at 25°C and 37°C. After collection the cells were stored at -80°C.

Cell collection:

Cultures from 24 hours and 120 hours were transferred to 50 ml conical centrifuge tubes and were then centrifuged at 15,000 rpm for fifteen minutes to form pellets. Both the 25°C and 37°C cultures from the mutant and wild type strains were washed three times with 1X TE buffer and centrifuged in the centrifuge at 15,000 rpm at 4 °C for fifteen minutes several times. The pellets were then weighed.

Collection of Conidia for RNA Extraction:

The conidia were collected after ten days of growth on PDA in petri dishes. Approximately four petri dishes for each strain were used to collect the conidia. The collection of conidia was done by pipetting 5 ml of phosphate buffered saline with Tween solution (PBST-100uL tween and 50 ml PBS) into each petri dish. The suspension of PBST containing conidia was then collected from each petri dish and placed into a 50 ml conical tube. The suspension was then spun for fifteen minutes at 2500 rpm on the centrifuge

(until a tight pellet was formed). The supernatant was discarded and the pellet was then re-suspended in 5 ml of phosphate buffered saline (PBS). The suspension was spun for fifteen minutes at 3400 rpm. The supernatant was discarded and an additional 5 ml of PBS was added. The suspension was spun again at 3400 rpm for fifteen minutes. The supernatant was once again discarded. The pellet was re-suspended in 1 ml ddH_20.

RNA Extraction:

RNA extraction was carried out using the RNeasy mini kit for Animal tissues and cells (Qiagen, CA) using the Qiacube (Qiagen, CA). Prior to starting the procedure, the cells were placed on ice and thawed (1 mold and 1 yeast for both mutant and WT). This was repeated for cells collected at 120 hours. In the tubes containing the cells, approximately 0.8 g of acid washed glass beads were added to each tube and 600µL of prepared RLT(containing guanidine thiocynate) + β-mercaptoethanol (acts as a lysis solution). The tubes were bead beat four times for 1 minute in a mini bead beater machine (Biospec) and then placed on ice for 1 minute. The tubes were then centrifuged at 4°C at 10,000 x g for a period of 5 minutes. This was done to separate the cells from the beads. The supernatant containing the cells was then transferred to fresh tubes. The samples were then spun once again at 10,000 g for 5-10 minutes to remove any pellets. The samples were then transferred to 2 ml Qiacube sample tubes. The amount of DNase mix was prepared keeping in mind the number of samples. The reagents used were RPE (for washing RNA), RW1**,** 70% ethanol and nuclease free water (Qiagen, CA). RNA purity was determined by analyzing the concentration using the Smart Spec spectrophotometer. After collection the samples were immediately stored at -80°C.

Additionally, RNA was extracted from conidia of both the wild type and mutant I242 at 25°C. The procedure for extracted RNA was the same as mentioned above with the exception of using 450µL of prepared RLT + β-mercaptoethanol. The samples were bead beat twice (instead of four times) for 1 minute in a bead better and then placed on ice for 1 minute.

DNA Digestion:

A Turbo DNase-free kit (Ambion, TX) was used to further purify the RNA and digest DNA that may still be present in the isolated RNA sample. Approximately 0.1 volume of 10X Turbo Dnase buffer was added to the RNA solution being purified. After which, 0.1 µL of Turbo DNase was added to the RNA solution. The tubes were centrifuged to ensure collection of the solution. The tubes were then incubated at 37°C for 20-30 minutes. 0.1 volume of resuspended DNase Inactivation Reagent was mixed well by vortexing the tubes. The tubes were then incubated at room temperature for 2 minutes after which they were centrifuged at 10,000 g for 2 minutes. The RNA solution was transferred to a new Eppendorf tube and the concentration of RNA was determined. Additionally, the A_{260}/A_{280} purity ratio values were determined. This was done to determine whether there was any protein or DNA contamination.

cDNA synthesis:

The iScript™ Select cDNA Synthesis Kit (Biorad, CA) was used. In the cDNA reaction mixture contained DNase treated RNA, 5X Buffer(containing dNTPs, magnesium chloride

and stabilizers), Oligo dTs, reverse transcriptase and nuclease free water were added (the volumes added were as per protocol). This mixture was incubated at 4 2°C for 1 hour and then stored at -20°C or was used immediately. The iScript™ Select cDNA Synthesis Kit (Biorad, CA) was used.

Calmodulin Reverse Transcriptase –Polymerase Chain Reaction:

To confirm the presence of cDNA and to check for any contamination, RT-PCR was carried out using housekeeping gene, Calmodulin (Calm) as the reference gene (PMAA_096920). To the reaction mixture cDNA, Calmodulin primers (Calm F & Calm R), GoTaq and nuclease free water were added. The volumes of the reagents were added as per protocol. Once the RT-PCR was complete, the samples were mixed with EZ-Vision 6X loading dye on a 2 % agarose gel to confirm the presence of cDNA and to see if there was any contamination. Once the presence of cDNA was confirmed the next procedure was to be carried out. The genomic DNA was found to be at 678 base pairs and the cDNA was found to be at 472 base pairs.

Table 1: Sequence of Calmodulin Primers

Calmodulin Primer Sequence	F 5’ TTG CAG GAC ATG TTG TGG TT 3’ R 5’ CGA TTC TTC CAT CAC CAT OC 3’

The PCR program used:

95°C for 3 minutes

95°C for 30 seconds
62°C for 30 seconds
72°C for 1 minute
} 35 cycles

72 °C for 7 minutes

Hold at 4°C

Temperature gradient:

To determine the correct temperatures at which the two sets of primers for FLBD (PM FLBD Start and PM FLBD End) work most effectively, a temperature gradient program was run on the thermal cycler using genomic DNA from the wild type. In the first temperature gradient PCR the temperatures ranged from 44- 60 °C. However, this was unable to provide any optimal temperatures for the PM FLBD start primers. A second temperature gradient PCR was run in which the range of temperatures were from 31-65.4°C. The start primers were found to be effective at 61°C and the end primers were found to be effective at 47°C.

Table 2: Sequence of *Penicillium marneffei flbD* start and end primers.

PMFLBD START	F 5' TCC AGG AAG AGG ACA ACA CC 3' R 5' CTT CGC TTG CGG TTC ATA TT 3'
PMFLBD END	F 5' AGA CGG CTT CAT CCT CAG AA 3' R 5' ATA GAT CCT GCG CCT CGA TA 3'

Primer and RNA testing:

An AcessQuick RT-PCR (PROMEGA) was performed on RNA extracted from both the yeast and mold from the wild type and mutant from 24 hour cell cultures (totaling four samples). This was repeated for cell cultures collected at 120 hours. The reaction mixture contained a 2 X Access Mastermix *(*with *Taq* DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers) , Calmodulin primer mix(100 uM), reverse transcriptase and nuclease free water. Depending upon the number of samples, the volumes of the solutions used were adjusted according to the protocol.. After the RT-PCR was completed the samples were mixed with EZ vision 6X DNA loading dye on a 2 % agarose gel to test the cDNA. If the correct bands were seen in all four lanes, the cDNA was found to be clean.

The cDNA from both the wild type and mutant (yeast and mold phases) was used for a second RT-PCR using the same reagents, but with FLBD start and end primers instead of Calmodulin primers. Genomic DNA was used as a control to ensure that the primers were functioning correctly. After mixing the solutions, the PCR tubes were placed in the thermal cycler. Once the program was complete, the samples were mixed with EZ vision 6X DNA loading dye and run on a 2% agarose gel. It should be noted that the concentrations of

cDNA were adjusted to ensure that each sample contained a total of 72 ug. This was done to ensure that level of expression observed would not be due to a difference in concentration.

PCR program for *flbD* start:

95°C for 5 minutes	
95°C for 30 seconds	35 cycles
61°C for 30 seconds	
72°C for 1 minute	
72 °C for 7 minutes	
Hold at 4°C	

PCR program for *flbD* end:

95°C for 5 minutes	
95°C for 30 seconds	35 cycles
47°C for 30 seconds	
72°C for 1 minute	
72 °C for 7 minutes	
Hold at 4°C	

Experimental cultures:

Wet mount slides were made using liquid cultures of inoculated SAB broth of *P. marneffei* F4 (wild type) and of the mutant strain I242. The cultures were grown at 25°C and 37°C. The slides were made of cultures grown for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours.

Additionally, slide cultures were made of both the wild type and mutant I242 using the method described by Harris (1986). For these cultures, PDA was used. The slide cultures were examined after 9 and 16 days of growth. Both types of slides were observed using an Olympus inverted phase contrast microscope. The images were taken using Spot-idea 5MP color mosaic camera. The images were analyzed using the SPOT-idea software (version 4.6). The samples were observed at 40x, 100x and 200x magnifications.

Results:

Primer testing:

Prior to starting any experiments involving testing of *flbD* start and *flbD* end primers with cDNA, the primers were tested using genomic DNA (gDNA) extracted from *P. marneffei* F4. PCR using the *flbD* primers was then carried out. Additionally, a temperature gradient PCR was carried out for each primer to determine at which temperatures the primer was most effective. For the *flbD* start primers, a temperature of 61°C was found to be most effective. For the *flbD* end primers, a temperature of 47°C was found to be most effective. The two sizes of the PCR products were approximately 678 base pairs for genomic DNA and472 base pairs for the cDNA. The results from the gradient PCR can be seen in Figures 5 and 6 below.

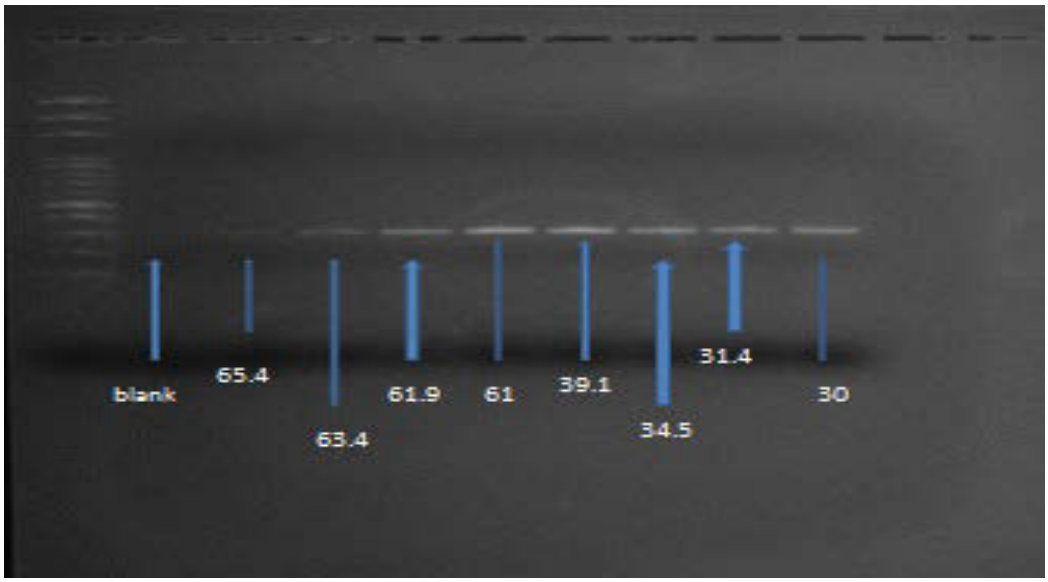


Figure 5: *flbD* end primers gradient PCR using gDNA from *P. marneffei* F4. The range of temperatures used for this particular gradient PCR range from 65.4 °C- 30 °C (g DNA ~678 bp).

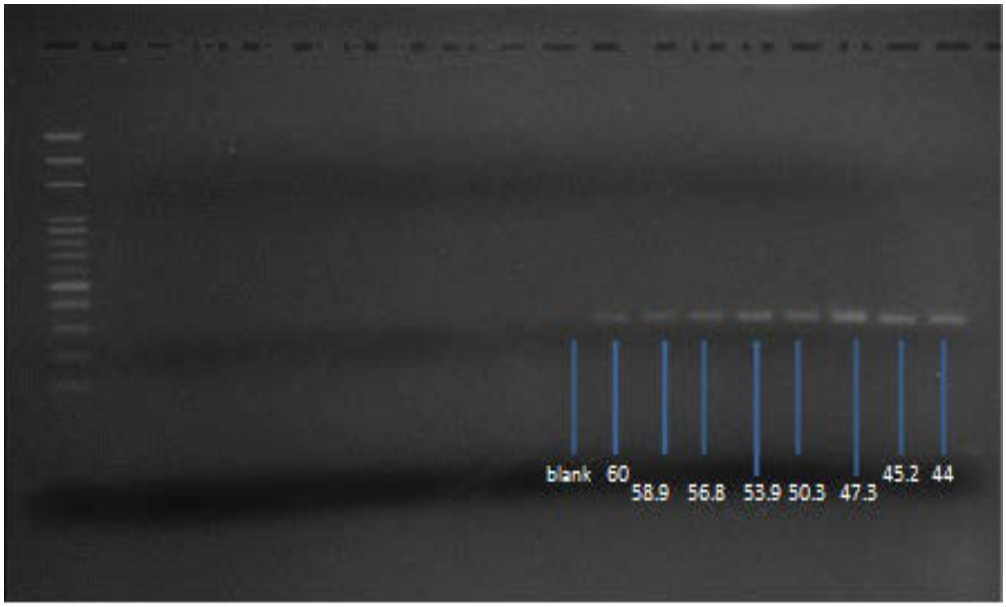


Figure 6: *flbD* end primers gradient PCR using gDNA from *P. marneffei* F4. The range of temperatures used for this particular gradient PCR range from 60 °C- 44 °C (gDNA~678bp).

Experimental cultures:

Penicillium marneffei wild type and I242 cultures were incubated at 25°C and 37°C in SAB; these were observed using an inverted phase contrast microscope at 24 hours and 120 hours. The samples were observed at 24 hour intervals from 24 hours-120 hours to see the progression in growth and development.

During the early stages of incubation the wild type at 25°C consists of hyphae which contain multiple branches. The hyphae were found to be thinner. At 120 hours, the network of hyphae can be best described as extensive. At 37°C, the wild type, at 24 hours consist of hyphae. The hyphae can be described as being shorter and thicker than the

hyphae observed at 25°C. During the later stages, the hyphae at 37°C transitioned into uni-nucleate circular yeast cells.

At 25°C, after 24 hours of growth, hyphae are observed in the mutant I242. These hyphae are similar to those observed in the wild type at this temperature. At 120 hours of growth, the network of hyphae was found to be quite extensive(Figure 7). At 37°C, at 24 hours of growth hyphae are observed. The hyphae observed at this temperature are similar to the hyphae observed in the wild type; they are shorter and thicker(Figure 8). At 120 hours of growth, unlike the wild type, hyphae are still observed. At 37°C, yeast cells are observed in the wild type as the process of arthrocondiogenesis is initiated after 96 hours of growth. This difference may be attributed to the mutation which is present in the promoter region of *flbD* gene. The *flbD* gene plays a role in conidiation and is thought to be over expressed in this mutant.

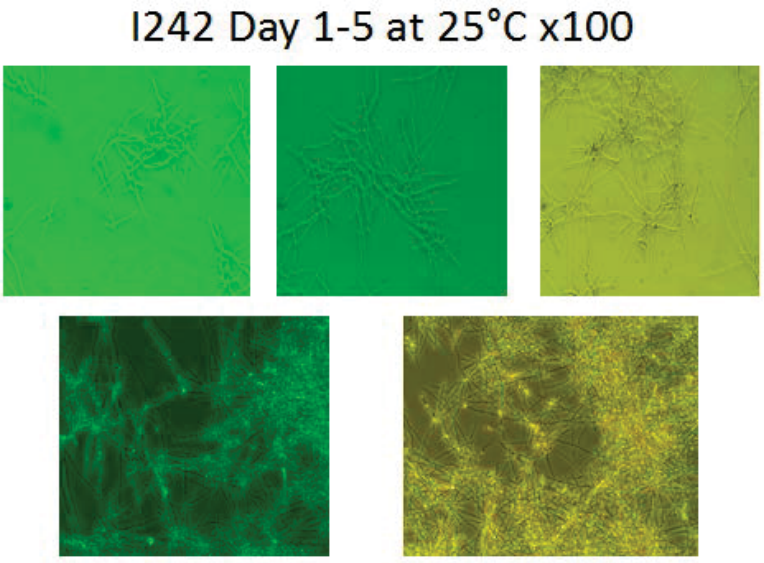


Figure 7: Mutant I242 grown in SAB broth at 25°C from Day 1-Day 5

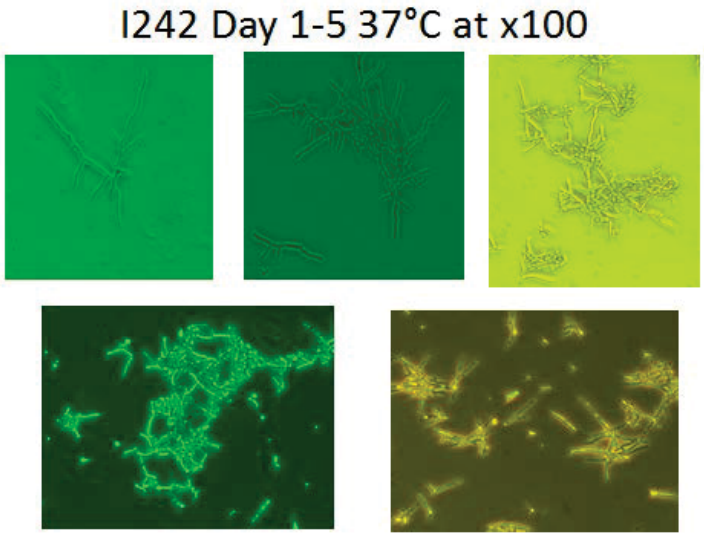


Figure 8: Mutant I242 grown in SAB broth at 37°C from Day 1-5.

Fungal slide cultures:

As previously mentioned, conidiation does not occur in liquid media. To obtain a better comparison of the structural and morphological differences between the wild type strain and the mutant I242 fungal slide cultures were made. The slides were examined at the 9th day of growth and the 16th day of growth. Figure 9 below shows the differences between the wild type and mutant I242 after 16 days of growth. As expected, chains of conidia are observed in both the mutant and the wild type at 25 °C. However, at 37°C unicellular yeast cells are observed in the wild type. In the mutant at this temperature, hyphal growth is observed.

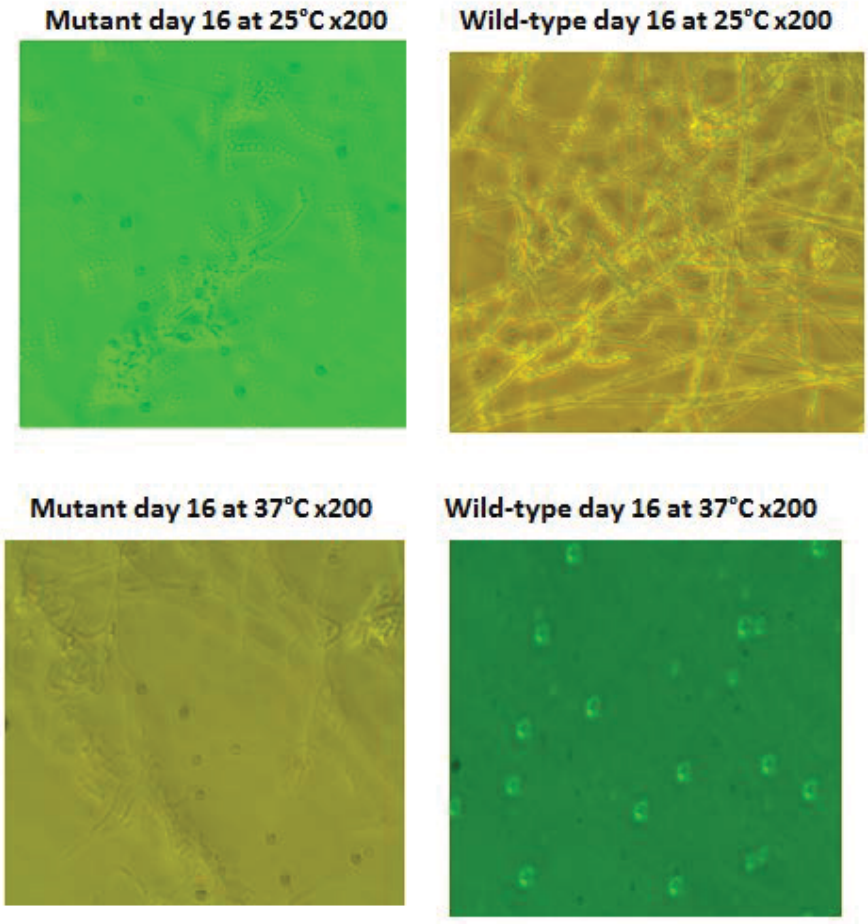


Figure 9: Comparison of wild type strain F4 of *P. marneffei* and I242 at 25°C and 37°C at x200 magnification.

RT-PCR:

RT-PCR was carried out on the cDNA derived from RNA; the RNA was extracted from wild type and mutant yeast and mold samples (24 hour, 120 hour and conidial samples). The cDNA was first tested with Calmodulin primers to ensure the presence of cDNA. Calmodulin was used as the house keeping gene. Once cDNA presence was confirmed, RT-PCR was carried out on the cDNA samples using the *flbD* start and *flbD* end primers. RT-PCR was run to see the differences in expression between the wild type and

mutant at 25°C and 37°C as well as at 24 hours and 120 hours of growth. RT-PCR was also used to determine difference in expression between the RNA collected from conidia of both the wild type and mutant I242. The results from the RT-PCR were analyzed by observing the products on a 2% agarose gel.

The results from the RT-PCR may be seen below in Figure 10. As mentioned previously, Calmodulin was used as the reference gene. The gDNA product is displayed at approximately 678 base pairs and the cDNA products are observed at 472 base pairs. The brightness of the bands observed in the cDNA from Conidia (both wild type and mutant) is less as compared to those at 24 hours and 120 hours.

With the *flbD* start primers no bands are visible with the cDNA extracted from the wild type at 24 hours and 120 hours. Nor are there are any bands visible with cDNA that was synthesized using RNA collected directly from the wild type conidia. However, bands are observed with the mutant I242 at 24 hours and 120 hours growth. Bands are also observed with the mutant conidial cDNA.

With the *flbD* end primers no bands are observed with the wild type at 24 hours in both the yeast and mold phases. However, at 120 hours, faint bands are observed in the wild type at both the yeast and mold phase. A faint band is also observed at the product from the wild type conidia at 25°C. In the case of the mutant I242, bands are observed at 24 hours, 120 hours as well as with the product from conidia.

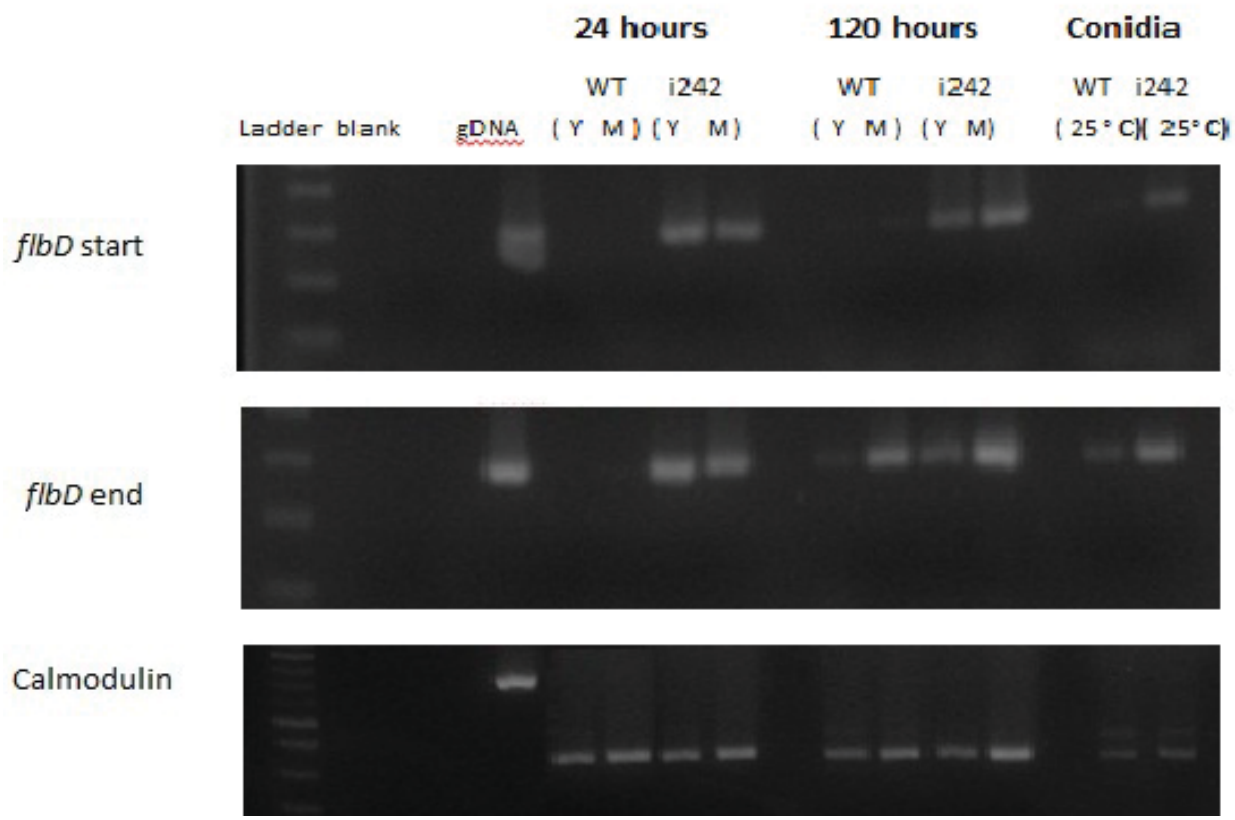


Figure 10: RT-PCR conducted with gDNA and cDNA samples with Calmodulin, *flbD start* and *flbD end* primers. RT-PCR with Calmodulin was done to ensure there was no contamination in the cDNA samples. (gDNA size~678 bp and cDNA size~472 bp)

Discussion:

As mentioned earlier, interest in *Penicillium marneffei* has increased over the past few decades. This is as a direct result of the high incidence of penicilliosis due to *P. marneffei* in immune-compromised individuals. Although cases of infection are mostly reported in individuals with suppressed immunity, cases have been reported in immuno-competent individuals. Although, more information is now available as to how the infection occurs in humans there is much about this organism that still needs to be studied. Similar to many other fungal pathogens, it is dimorphic; at 25°C it exists as a mold and at the 37°C it exists as yeast cells. The morphology observed within the host tissue is that of unicellular yeast cells. By increasing our understanding of the mechanisms involved in dimorphism and conidiation we may be better able to counter the pathogen. It is thought that the mechanism for conidiation has been more or less been conserved in filamentous fungi.

brlA has been identified as being a key regulator for conidiation. *brlA* mutants with loss of function in *A. nidulans* were found to form structures that were similar to conidiophores (known as bristles). However, they formed no vesicles, metulae or phialides; conidiation was also not observed in these mutants (Adams et al., 1998). In *A. fumigatus*, asexual development did not occur in mutants with deletion of *brlA* (Mah and Yu, 2006). It was determined that *brlA* expression in these two *Aspergilli* species is needed for conidiation to occur.

abaA was found to regulate the differentiation of the phialides (Sewall Et al., 1990b). Furthermore, it was determined that over expression of this gene did not result in formation of conidia (Mirabito et al., 1989; Adams and Timberlake, 1990). A homologue of

this gene was found in *Penicillium marneffei.* The *P. marneffei* homologue, *abaA,* functions during both conidiation and dimorphic growth (Borneman et al., 2000).

The final gene in the regulatory pathway for conidiation is *wetA* (wet-white). The mutants of this particular gene resulted in the formation of conidia that was colorless that autolyzed within a few days to give liquid droplets (Clutterbuck, 1969). It was also determined that the *abaA* is needed for the activation of wetA. This was determined in a study in which *abaA* overexpression activated *wetA* in the absence of wild type *brla* (Mirabito et al;, 1989). A homologue for *wetA* in *Penicillium chrysogenum* suggests that systems for control of spore formation and development have been conserved in *Aspergillus* and *Penicillium* (Prade and Timberlake, 1994; Roncal and Ugalde, 2003).

Several upstream regulators of asexual development in *A. nidulans* have been determined. These are *fluG, flbA , flbB, flbC, flbD* and *flbE*. As mentioned previously the order of activation of these genes are *fluG → flbC → flbE → flbD → flbB.* In *A. nidulans*, *flbB, flbC* and *flbD* are thought to function as putative transcription factors (TF). These are thought to act as DNA binding proteins which are activate other developmental regulators such as *brlA* (Adams et al., 1998). Further studies did in fact determine that *flbB, flbC* and *flbD* activate *brlA* expression (Etxebeste et al., 2008, 2009; Garzia et al., 2009 and 2010; Kwon et al., 2010).

In *P. marneffei*, the activation of *abaA, brlA* and *wetA* were found to be part of the key transcriptional regulatory pathway for conidiation. *brlA* is in turn repressed by G*asA*. *GasA* was found to be a promoter of vegetative growth (Zuber et al., 2002). These transcription factors are thought to play a role in the activation of different genes involved

with morphogenesis. In *P. marneffei,* it is proposed that the GasA subunit plays a role in suppressing *brlA* during yeast growth (Zuber at al., 2002). Additionally, *abaA* feedback was found to activate expression of *brlA* in hyphal cells but not yeast cells. This evidence suggests that the elements that are part of the regulatory pathway for conidiation may also be key players in dimorphic switching.

The mutant I242 of *Penicillium marneffei* has a known mutation in the promoter region of the gene *flbD* (Kummasook, 2010). The aims of this research were to study the expression of *flbD* in the mutant and the wild type in an attempt to gain a greater understanding of the function of *flbD* gene and the role it may play in conidiation. The results show that, under conditions that do not favor conidiogenesis (growth in broth), *flbD* is expressed by strain I242, but not the wild type. This observation supports our hypothesis.

The cDNA synthesis from RNA collected from both the mutant and wild type was tested to see if it was free of DNA contamination. The house keeping gene Calmodulin was used. It was chosen as the reference gene as it present at fairly constant levels and is present in both the yeast/mold phases. Another advantage to using Calmodulin as the housekeeping gene was the difference in size of the genomic DNA and complementary DNA products (678 base pairs and 472 base pairs respectively).

It should be noted that the concentrations of the cDNA were adjusted to ensure that the concentration of each sample was 12ug/uL. For RT-PCR with each of the three primers (Calmodulin, *flbD* start and *flbD* end) approximately 6uL was used; the cDNA concentration in each sample was thus 72ug. This was done to ensure that any difference that would be

observed would be due to differences in expression rather than a difference in concentration.

It was proposed that as the mutation occurs in the promoter region of the *flbD* gene, it may prevent binding of the repressor *sfgA*. *sfgA* was found to be a repressor of *fluG* in *Apergillus nidulans* (Seo et al., 2003, 2006). It is thought that *sfgA* may be a transcription factor; it is a negative regulator of conidiation and may activate a series of repressors that suppress conidiation. If binding of *sfgA* does not occur it would result in continuous conidiation as *flbD* would be over expressed.

***flbD* start:**

By examination of the results of RT-PCR with *flbD* start primers from 24 hours of growth during both mold and yeast phases (of both wild type and mutant I242), it was determined that there were no bands visible with the wild type at 24 hours of growth. However, with the mutant at 24 hours bands are observed in both the yeast and mold phases. At 120 hours of growth, in the wild type once again bands are not observed. At 120 hours of growth, in the case of the mutant I242 bands are observed in both the yeast and mold phases. This is consistent with what was proposed that as *sfgA,* the negative regulator of *flbD*, is unable to bind resulting in over expression of *flbD*. *flbD* may continue to activate *brlA* and conidiation will continue to occur in the mutant. Furthermore, conidiation does not occur in the wild type yeast. The absence of bands in the wild type mold be attributed to the fact that conidiation may not occur within 24 hours of growth. Additionally, with the samples collected from the conidia, a band is observed with the mutant I242 but not with the wild type.

***flbD* end :**

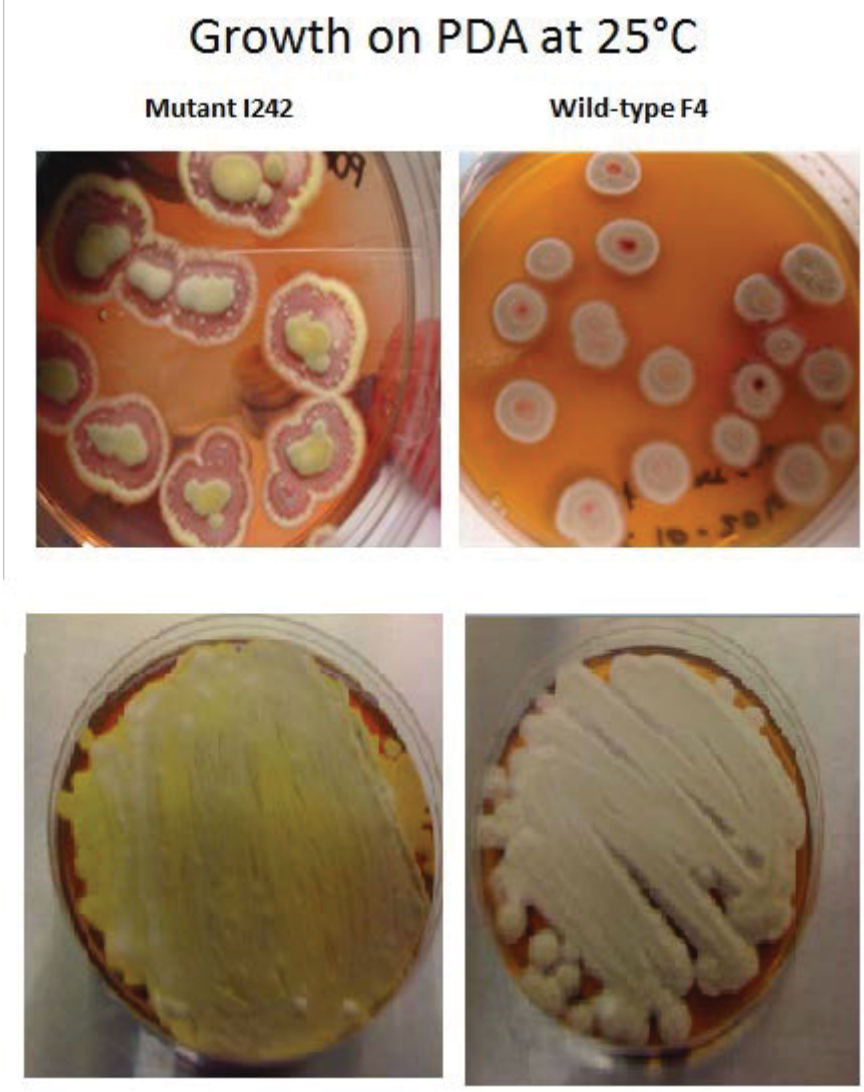
The results from RT-PCR with the *flbD* end primers from 24 hours of growth during both mold and yeast phases of the wild type no bands observed (similar to the *flbD* start primers). This is consistent with what is expected as conidiation does not occur in the wild type(mold) as early as 24 hours. With the mutant I242 at 24 hours of growth bands are observed with both the yeast and mold phases. At 120 hours of growth, bands are observed in both wild type yeast and mold phases. However, as expected the intensity of the bands in the mold phase in the wild type is more pronounced. This can be attributed to the fact that conidiation does not occur in the wild type in the yeast phase. With the mutant at 120 hours, bands are seen in both yeast and mold phases. This appears to be consistent with what was proposed; over expression of *flbD* occurs. It was also expected that conidiation would continue to occur at the yeast phase of the mutant which is unlike the wild type for this reason. Again as with the *flbD* start primers, a band is observed with the conidial cDNA in the mutant. A faint band is observed with the wild type. This can be attributed due to the mutant perhaps producing more conidia than the wild type even at 25°C. One would expect to see a PCR product in the wild type mold phase at 120 hours. However, a possible reason for the lack of bands in both the start and end primers of the wild type mould at 120 hours of growth could be that the cultures were grown in broth and conidia are not formed in broth in the wild type. However, bands are visible for the mutant at both yeast and mold phases of growth at 24 hour and 120 hours of growth respectively..

The differences in results with the start and end primers can be attributed to the fact that there may be a different expression pattern within the same gene. The difference may be due to one region of the gene being more active than the other during certain times. This can be highlighted by looking at the contrasting results from 120 hour growth of the wild type (both yeast and mold phases) in the two sets of *flbD* primers. Bands are only observed with the *flbD* end primers. It would be beneficial to look at expression at different time intervals as well as under varying stresses to determine the expression profile of the start and end regions.

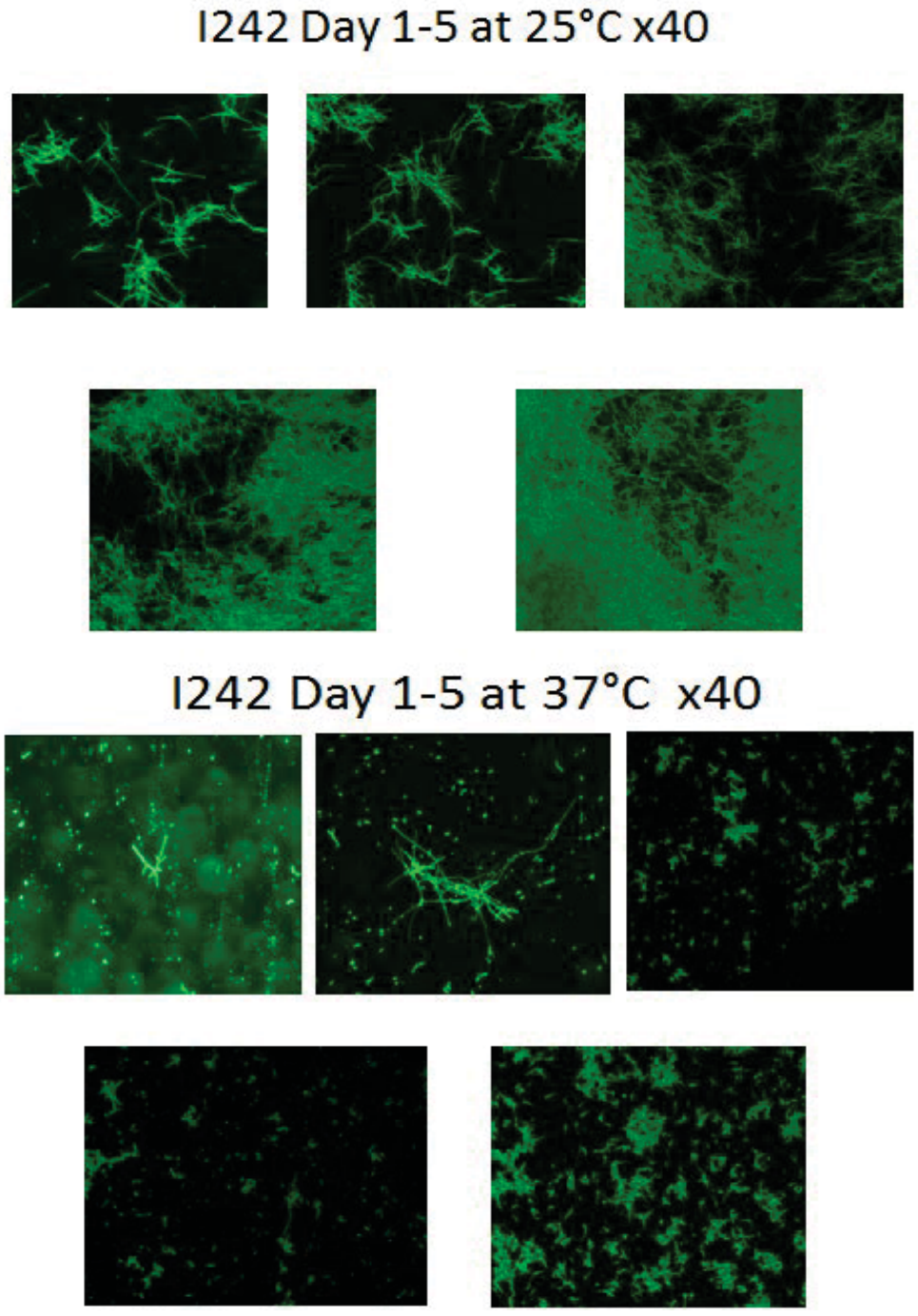
By analyzing the results it can be deduced that *flbD* does in fact play a key role in conidiation in *Penicillium marneffei*. A difference was in expression was seen in both the wild type and mutant I242. It may be due to the continued indirect activation of *brlA* by *flbD*; *flbD* directly affects *flbB* expression which activates *brlA.* Additionally, the repressor or homologue of the repressor sfgA plays a role in the negative regulation of conidiation in *P. marneffei.*

As mentioned earlier, the six upstream regulators of conidiation have been identified in *A. nidulans.* The roles of these genes have not yet been fully determined in *P. marneffei.* Further investigation of each of these genes in *P. marneffei* is needed; it would be interesting to see whether the manner in which they regulate conidiation is similar to *Aspergillus nidulans* and *Aspergillus fumigatus*. It would also be useful to look at *flbB* in future studies and its role in conidiation- whether or not *flbD* is able to bypass the need for *flbB to* activate *brlA.*

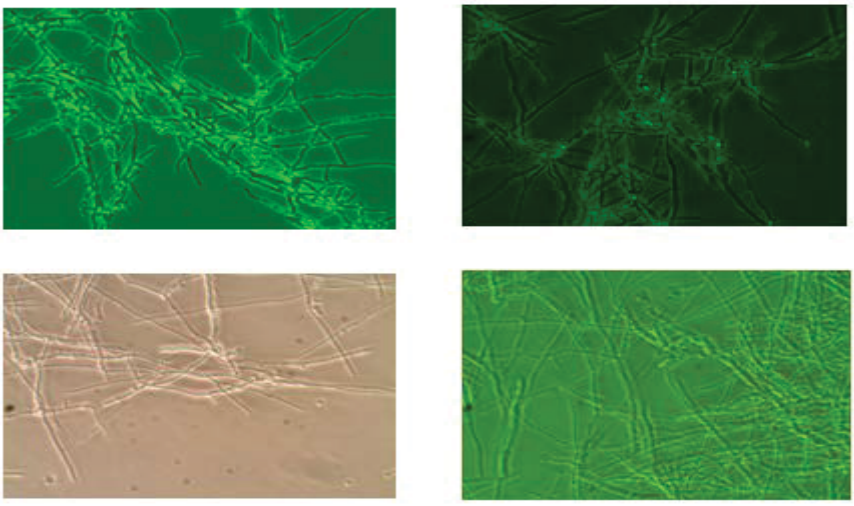
Appendix:



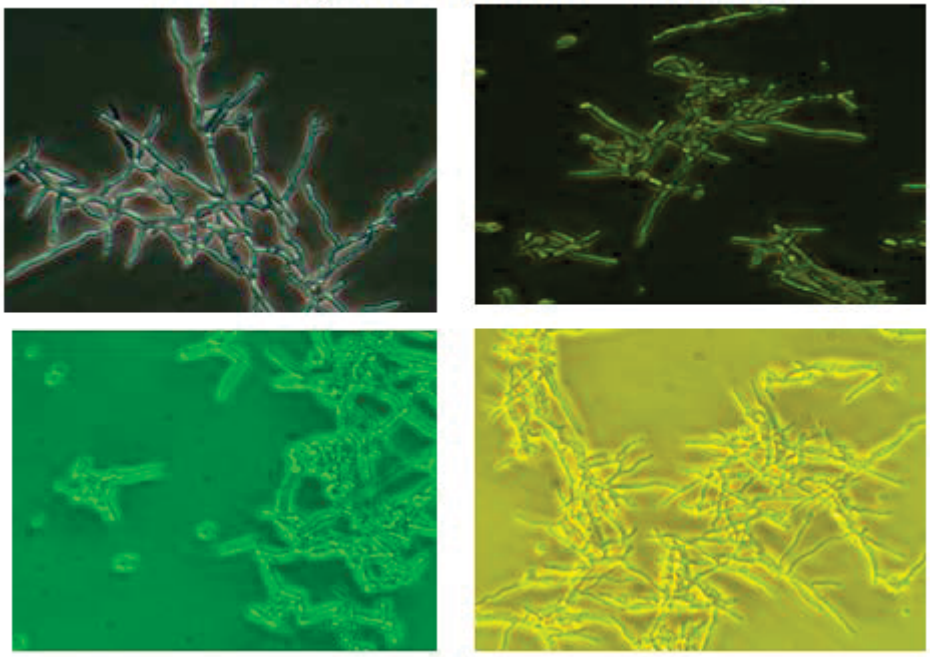
SAB broth morphology:



I242 Day 2-5at 25°C x200



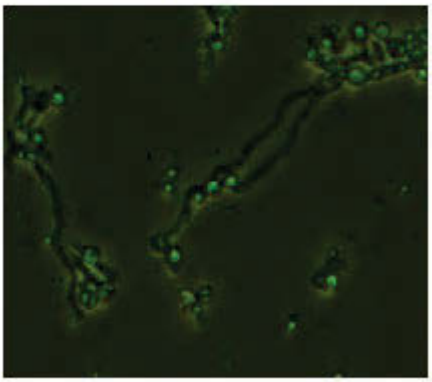
I242 Day 2-5 at37°C at x200



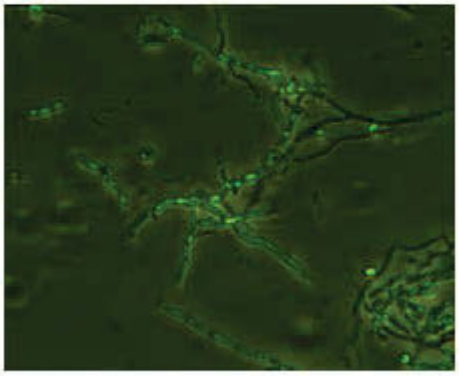
Fungal slide culture:

Comparison of I 242 and WT

Mutant day 9 at 37°C at x200

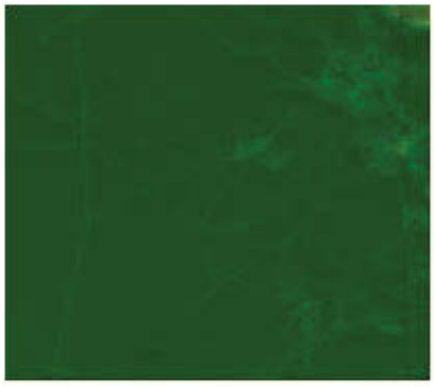


Wild-type day 9 at 37°C at x200



Comparison of I242 and WT

Mutant day 9 at 25°C at x100



Wild-type day 9 at 25°C at x100

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