Analysis of a *ufdB Penicillium marneffei* Mutant Generated by *Agrobacterium tumefaciens*-Mediated Transformation

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ABSTRACT

Penicillium marneffei is a notorious, medically pertinent fungal pathogen responsible for causing penicilliosis within immunocompromised individuals, particularly those with HIV/AIDS. Existing as a mold at 25°C and yeast at 37°C, it is the only *Penicillium* species known to undergo a temperature-dependent dimorphic switch. Recently, *Agrobacterium tumefaciens*-mediated transformation (ATMT) was used to introduce randomly integrated T-DNA fragments into the *P. marneffei* genome. This study sought to genotypically and phenotypically characterize mutant I189. An inverse PCR protocol was employed to recover the DNA sequences flanking the T-DNA insertion site. BLAST analysis revealed an interruption of a putative ubiquitin fusion degradation protein (UfdB) mRNA. Orthologous in the human pathogen *Aspergillus fumigatus* as well as model yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae,* this protein has been documented to tag abnormal stress-induced proteins for degradation. Current investigations of *ufdB* in *P. marneffei* mutant I189 show that fungal growth is significantly diminished in the presence of nutrient limitation although a full mRNA transcript is being produced. However, exposure to Congo Red, sodium dodecyl sulfate (SDS), and heat stress had no effect on cell viability.

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CHAPTER I INTRODUCTION

1.1 Background

Penicillium marneffei is an opportunistic, ascomyceteous fungal pathogen that undergoes morphological changes once subjected to particular thermal conditions. When cultivated at room temperature, or 25°C, it proliferates as a filamentous, multinucleate mold that produces conidia. At 37°C, *P. marneffei* partakes in binary fission to produce yeast cells (1). It is this latter mode of development that is commonly observed within the lung, liver, spleen, and bone marrow of immunocompromised individuals (2). This phase transition that results in the condition known as dimorphism allows previously non-pathogenic multicellular hyphae to ravage its host in widespread disease in the form of uninucleate yeast. To date, the gene or genes enabling this temperature dependent process have yet to be fully revealed.

1.2 Ecology and Epidemiology

In 1956, investigators first isolated *Penicillium marneffei* from the liver of a captive Chinese bamboo rat, *Rhizomys sinensis* (3). Upon autopsy following the unexpected, spontaneous death of the rat, an inflamed spleen and nodules were detected within the rodent. Tissue isolates were sent to the Pasteur Institute of Indochina to be cultured. The resulting fungus that grew in culture was characterized as a new species and appointed the name *Penicillium marneffei,* after director Dr. Hubert Marneffe (4). Deng and associates assessed bamboo rats from another region of China in 1986. The captured rodents were of the *Rhizomys pruinosus* species. Although the rats displayed no

signs of illness, *P. marneffei* was obtained from multiple organs at the time of death. Taken together, the investigators decided that the source of infection was from nature (5).

It was not long before human case studies of penicilliosis due to *P. marneffei* emerged. The first infection occurred when Segretain unintentionally punctured his finger with a needle containing *P. marneffei* in 1959. Shortly thereafter, he suffered from axillary lymphadenopathy. The fungus was isolated from a nodule that surfaced at the site of inoculation. Segretain was restored back to health after a 30 day treatment of oral nystatin (6). In 1973, Di Salvo *et al.* reported the first case of naturally occurring penicilliosis due to *P. marneffei*. A 61-year-old clergyman had undergone a splenectomy for Hodgkin's disease which later revealed the presence of *P. marneffei*. He had previously resided in Southeast Asia (7). Eleven years later, in 1984, a second case was noted in a 59-year-old man who traveled to the Far East. He experienced repeated and persistent bouts of hemoptysis. Believing it was symptomatic of bronchitis, partial removal of the lung displayed many nodular granulations. Further examination of the recovered lung tissue exhibited uninucleate *P. marneffei* yeast cells (8).

There were five more incidents of disease described that same year. Patients, who ranged from 6 to 50-years-old, suffered from fevers and swollen lymph nodes. All had lived in various regions of Thailand. Those in the early stages of infection were able to be successfully treated with Amphotericin B (9). In the next seven years, over 30 additional cases were reported, from Hong Kong to the Guangxi region of southern China (10-22).

Sudden onset of the HIV/AIDS epidemic in Asia substantially increased the incidence of human penicilliosis during 1988. Those infected by this opportunistic

pathogen were not only natives, but individuals traveling to Southeast Asia from Australia (23), France (24-27), the Netherlands (28, 29), the United Kingdom (30-32), and the United States (33). Typically, the patients were homosexual males, while others were drug abusers. Penicilliosis due to *P. marneffei* was commonly seen in those afflicted with HIV, and usually indicated ongoing infection. In 1989, the first of many cases emerged regarding AIDS patients indigenous to Southeast Asia. Reports involving heterosexual men from Thailand engaged in relationships with prostitutes surfaced in 1991 (34) and 1992 (35). This infection became the third most common illness observed in patients with AIDS (36). Reports regarding this organism continue to be published with the latest incident occurring in a 14-year-old Thai girl (37).

1.3 Morphology and Mycology

Penicillium marneffei thrives as a mold yielding conidia when grown at 25°C. It is under these same conditions that the conidia engorge and expand to create germ tubes. As the germ tube generates hyphae, septation promotes individual cell formation. Primary stalk cells are produced by special vegetative mycelia once hyphal development is achieved. These septate cells form secondary stalks that generate uninucleate metulae and phialides. This is accomplished through budding. Metulae, which are first to germinate, make way for phialide formation. Basipetally, phialides bring forth and bear conidia. As this occurs, younger and newer spores relocate older spores located toward the tip of the phialide. On synthetic media, a fuzzy texture is observed (38). Spore pigment may vary from fawn colored to green depending on the strain and/or location from which the isolate is attained. Also, a soluble red pigment is released into the

surrounding agar (39). Upon increase of the temperature, *P. marneffei* undergoes phase transition, thereby altering its morphology. Conidia harvested at 25°C begin to couple nuclear and cellular division cycles at 37°C. Substantially branched hyphae that are wider and shorter than those grown at 25°C proliferate after the first 48 hours. These well-defined cells, which contain a single nucleus and are surrounded by double septa, are now called pre-arthroconidia. Sequentially, arthroconidia are formed after pre-arthroconidia separate and release individual cells. Arthroconidia then divide by means of binary fission, permitting the generation of yeast cells. The cells continue to quickly split and elongate until all transition into the yeast phase. When artificially cultured, colonies are glossy in appearance. This thermally driven, mold- to-yeast conversion has captivated many researchers who wish to discover the genes that govern *P. marneffei*'s morphogenesis (40).

1.4 *Agrobacterium tumefaciens*-Mediated Transformation

Agrobacterium tumefaciens-mediated transformation (ATMT) is a highly effective technique used to generate a wide range of transformants through targeted and random mutagenesis. This system has been utilized to successfully disrupt and mutate the fungal genomes of mushrooms, plants, and yeast (41-43). ATMT permits the use of many substrates including conidia and mycelia, therefore eliminating the need for protoplasts (44). Stably integrated, these transformants have allowed investigators to carefully study and examine all aspects of gene function.

Agrobacterium tumefaciens, originally classified *Bacterium tumefaciens,* is a Gram-negative bacterium that produces crown gall tumors in the tissue of plants (45, 46).

This soil-borne pathogen utilizes transfer DNA (T-DNA) and a virulence (*vir)* region on its tumor-inducing (Ti) plasmid to induce abnormal, uncontrolled growth (47). Wounded and lacerated plants release phenolic compounds, like acetosyringone, that stimulate *A. tumefaciens'* virulence proteins VirA and VirG. This two-component system, responsible for T-DNA transport, initiates sequential expression of dormant *vir* genes (48, 49). In the absence of AS, certain monosaccharides have been shown to also induce *vir* gene expression. ChvE, a chromosomally encoded virulence protein, works with VirA to enhance *vir* induction in the presence of these sugars (50). A single-stranded copy of T-DNA, termed the T-strand, is generated by the products of *virC* and *virD*. VirD1 and VirD2 collaborate to precisely nick the lower T-DNA 25-base pair border repeat (51-54). VirC1 serves to improve T-strand formation when VirD1 and VirD2 concentrations are dwindling (55). It is this incision that liberates a single T-DNA strand. At this time VirD2 and VirE2, a single-stranded DNA binding protein, associate with the T-strand to form the T-complex. Each shield the T-strand from nucleases and preserve its unfolded nature (56, 57). This complex is transported into the host cell with the aid of VirB11 and VirD4. VirB11 is a protein kinase and ATPase that creates an opening for transport and provides energy during the import process. Meanwhile, VirD4 arbitrates communication between the T-complex and the VirB pore (58). After entering the host, the T-complex transmits signals to locate the nucleus. Once within the nucleus, T-DNA integrates the genome and completely transforms the cell. The distinct method by which this occurs is still unknown (59-61).

1.5 T-DNA Binary Vector Systems

Early attempts to insert genes of interest (GOI) into T-DNA were laborious and time-consuming. The Ti plasmid was substantially large and challenging to engineer in preferred host, *Escherichia coli*. Also, the T-DNA region was not comprised of restriction sites that would enable cloning of GOI (62-64, Fig 1A). In 1983, Hoekema, *et al.* established a new technique of transferring genetic material into plants, where the need for a single, intact Ti plasmid was no longer needed. This group discovered that the T-DNA and *vir* portions of the Ti plasmid can be placed on two individual plasmids (65). T-DNA is situated on the binary vector containing the origin(s) of replication, GOI, antibiotic-resistance genes utilized to confirm the presence of the vector within the bacterium, and a selectable marker. This marker verifies plant transformation. The *vir* genes, located on the *vir* helper, are retained on their own replicon within *A. tumefaciens*. Once the two plasmids interact, routine integration of the plant genome occurs (Fig. 1B).

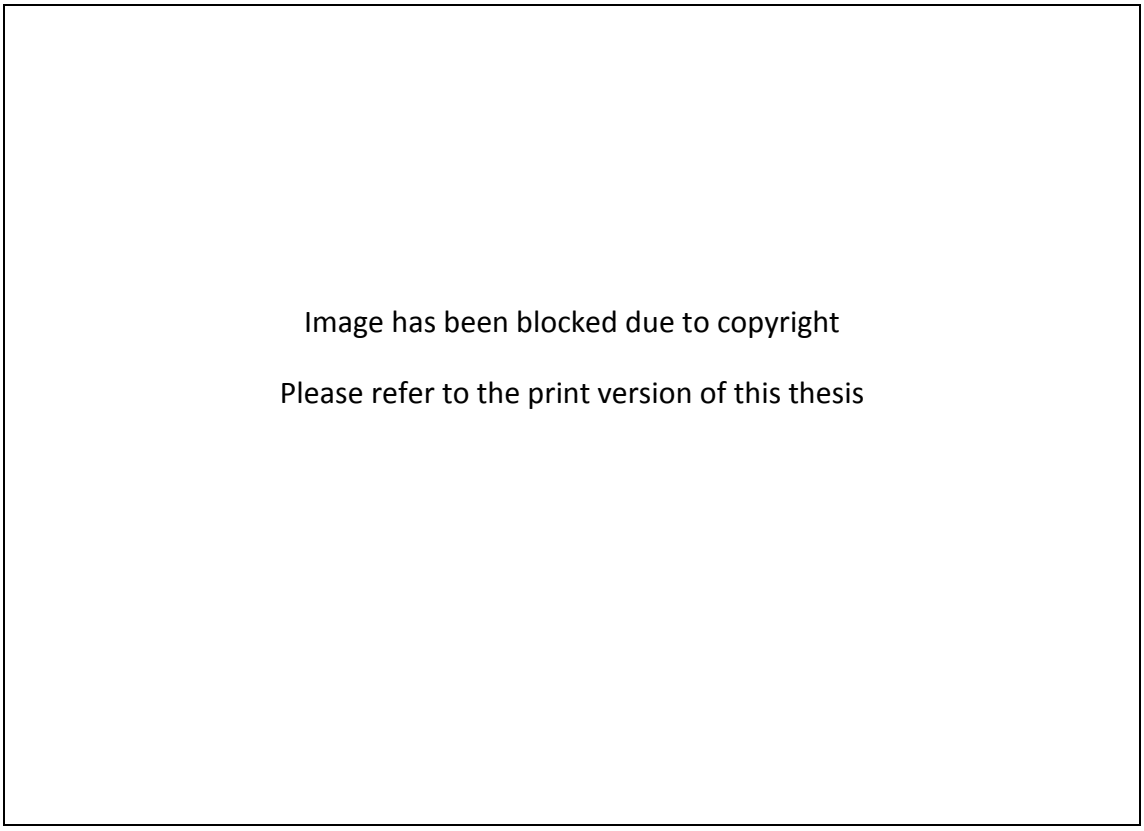

Figure 1 Schematic diagram of A) co-integration/exchange systems and B) T-DNA binary vector systems to introduce genes into plants using *Agrobacterium*-mediated genetic transformation (reproduced from Lee and Gelvin, 2008 , with permission from the authors (64; Appendix I)).

1.6 Mutant Generation and Formation in *P. marneffei*

In 2008, the first report of ATMT in *P. marneffei* was published. Zhang *et al.* combined different *A. tumefaciens* strains and binary vectors to achieve the most efficacious match. Strain EHA105 containing vector pBI129A was shown to successfully generate the most transformants. Co-cultivation with *P. marneffei* germlings for 4 to 5 days on induction medium led to 82 transformants. The medium was supplemented with acetosyringone. *P. marneffei* does not produce this phenolic compound, and over 90% of colonies grown without it were false-positives containing no gene insertion. Nitrocellulose filters were placed over the co-cultivating mixture to select

and screen mutants. Southern blot analysis revealed that each copy was randomly introduced into the genome, with 87.5% of them being single-insertion events. Thermal, asymmetric, interlaced-polymerase chain reaction (TAIL-PCR) amplified DNA flanking the insertion site, further confirming that insertion was arbitrary, as each sequence differed from one another (66). Collectively, this study established the use of ATMT to develop, assess, and examine the gene function of *P. marneffei* mutants.

More recently, Kummasook *et al.* explored ways to improve upon this efficient method. *A. tumefaciens* AGL1-pUPRS0 was co-cultivated with pre-germinated from *P. marneffei* conidia for 36 hours at 28°C. AGL1 contains selectable markers for carbenicillin and rifampicin, while pUPRS0 possesses bleomycin, kanamycin, and neomycin resistance genes. These optimal conditions produced close to 12,000 transformants. Of twenty arbitrarily isolated transformants, 95% were estimated to contain a randomly integrated, single T-DNA copy. Cost-effective cellophane sheets were used for screening instead of nitrocellulose membranes. Eight mutants isolated at 37°C were evaluated at 25°C for morphological variances. Their sequences, amplified by inverse PCR instead of TAIL-PCR, were compared against the fungal genomes in the GenBank database. Among others reported, *gasC* and *stuA* mutations were characterized (67). These investigators have performed and presented an enhanced ATMT protocol that increased the yield of stable *P. marneffei* transformants with single T-DNA insertions.

CHAPTER 2 SPECIFIC AIM

As previously mentioned, almost 12,000 mutants were produced through ATMT by Kummasook *et al*. A vast majority of them are yet to be assessed. This investigation seeks to genotypically and phenotypically characterize *Penicillium marneffei* mutant I189. Unlike the wild type, this mutant exhibits yellow pigmented conidia at 25°C and does not release brown pigment into the agar when cultivated at 37°C. Fungal melanins have been documented to protect fungi against lysis and extreme temperatures as well as contribute to virulence and pathogenicity when present within a host organism (68, 69). The gene disrupted by ATMT may encode a protein involved in melanin biosynthesis.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Media

Unless otherwise stated, all chemicals and culture media utilized during experimentation were acquired from Amresco LLC (Solon, Ohio), BD Difco (Franklin Lakes, New Jersey), Integrated DNA Technologies (Coralville, Iowa), Pharmco-AAPER (Philadelphia, Pennsylvania), Sigma-Aldrich (St. Louis, Missouri), or Thermo Fisher Scientific (Pittsburgh, Pennsylvania). The brain heart infusion agar (BHIA), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) and broth (SDB), and water agar were prepared in accordance with the manufacturer's instructions using distilled deionized water.

3.2 Methods

3.2.1 Maintenance of Strains and Environmental Conditions

Penicillium marneffei strain F4 (wild type; CBS 119456) was isolated from a patient diagnosed with AIDS in Chiang Mai, Thailand in 1999 and was kindly donated to Youngstown State University (1). This strain and the ATMT-generated mutant I189 were cultured and cultivated at 25°C and 37°C on MEA or BHIA in 25 m² cell culture flasks with vented caps. Glycerol stocks were maintained and frozen at -80°C.

3.2.2 Verification of T-DNA Integration

The T-DNA plasmid vector utilized by Kummasook *et al.* contained a resistance marker for bleomycin, an antibiotic cancer drug (Appendix A; 70). To validate whether recombination occurred and T-DNA integration took place, wild type and I189 isolates were streaked on BHIA supplemented with 4 micrograms per milliliters of bleomycin. The plates were incubated 37°C and assessed for yeast growth after 3 days.

3.2.3 Harvesting of Conidia for Broth Inoculation

Ten milliliters of sterile distilled deionized water was added to cell culture flasks containing the fungal strains of interest. A cell scraper was inserted into each flask and used to carefully remove the conidia adhered to the surface of the agar. These suspensions were added to an assembled screen cap (Bio-Rad; Hercules, California) containing sterile glass wool (Corning; Corning, New York) within it, affixed to the mouth of a sterile 50 ml conical centrifuge tube (71), and placed on ice. The tubes were centrifuged at 1,000 rpm for 30 seconds in a Beckman GPR centrifuge (Beckman Coulter; Brea, California) refrigerated to 4°C. The solutions were quickly vortexed, serially diluted, and conidia were counted using an improved Neubauer hemacytometer. Conidia, at a final concentration of 1×10^6 conidia per milliliter, were added to 50 ml of sterile SDB placed in 250 ml Erlenmeyer flasks. The liquid cultures were deposited in 25°C and 37°C water baths, shaking continuously at 120 rpm, for 48 hours. The cells were placed in 50 ml centrifuge tubes and spun down for 15 minutes at 4,000 rpm at 4°C. The supernatants were removed and 800 microliters of each strain at each temperature

were placed into four labeled 1.5 ml microcentrifuge tubes. All were spun down for 2 minutes at 12,000 rpm, decanted, and frozen at -20°C for molecular analyses.

3.2.4 Genomic DNA Isolation

To molecularly examine each strain, one loopful of conidia from the wild type and mutant was inoculated in 50 ml of sterile SDB contained within 250 ml Erlenmeyer flasks. The flasks were placed in a 25°C water bath shaking at 135 rpm. After 24 hours, they were moved to a shaking 37°C water bath for 40 hours. The suspensions were added to 50 ml conical centrifuge tubes and centrifuged at 4,000 rpm for 10 minutes at 4°C. The supernatants were discarded and approximately 25 ml of ice cold 0.6 M magnesium sulfate was added. They were spun down as previous described and decanted. Up to 25 ml of osmotic buffer (magnesium sulfate heptahydrate, sodium phosphate buffer, pH 7.0) was added to the soft pellets, spun down for 5 minutes, and the supernatant was removed. This wash was repeated twice. Each strain was resuspended in 10 ml of osmotic buffer and added to two new 250 ml Erlenmeyer flasks. Lysing enzymes from *Trichoderma harzianum* (0.12 g/ml) and 1 ml of bovine serum albumin (BSA;12 mg/ml) were combined together, added to10 ml of each culture, and incubated in a 37°C water bath shaking at 100 rpm for an hour and fifteen minutes. The samples were placed in 50 ml centrifuge tubes, spun down, and decanted. Each was washed twice with 10 ml of ST buffer (D-sorbitol, 1M Tris-HCl, pH 7.5), centrifuged, and the supernatants were removed. Once resuspended with 5 ml of lysis buffer (10 MM Tris, 1 MM EDTA, 1% SDS), the mixtures were vortexed and incubated at 65°C for one hour. They were vortexed every thirty minutes, or twice, during incubation. One and a half

milliliters of 5 M potassium acetate was added to the suspensions, mixed by inversion, and were left to rest on ice for one hour. The solutions were centrifuged for 30 minutes at 3,500 rpm at 4°C. The supernatants were collected and placed in new 15 ml centrifuge tubes. Three milliliters of phenol:chloroform, pH 6.7/8.0 was added to the samples, vortexed, and the tubes were spun down for 30 minutes. The supernatants were transferred to new 15 ml centrifuge tubes and RNase A (50 µg/ml) was added. The tubes were incubated at 37°C for one hour without shaking. Phenol:chloroform was again added as previously mentioned and centrifuged. The supernatants were carefully removed and placed in 50 ml centrifuge tubes. Two volumes of absolute ethyl alcohol was slowly added to the tubes and mixed. They were incubated for 12 hours at -20°C. Supernatants were removed and discarded after the tubes were centrifuged for 45 minutes at 4,500 rpm. The resulting pellets were washed twice with 1 ml of 70% ethyl alcohol and spun down. Once decanted, the pellets were left to dry for 1-2 hours at room temperature. The DNA was resuspended in 500 microliters of nuclease free water and refrigerated overnight. The concentration and integrity of the eluted DNA was assessed using the Smart Spec™ Plus spectrophotometer (Bio-Rad). All samples were stored at -20°C.

3.2.5 Restriction Endonuclease Digestion

Wild type and mutant genomic DNA were acquired from -20°C storage, defrosted to room temperature, and placed on ice. Restriction endonucleases *Nde*I, *Xho*I, *Bam*HI, or *Xba*I (Fermentas; Glen Burnie, Maryland) were combined with appropriate concentrations of nuclease-free water, 10X FastDigest buffer, and DNA, in accordance

with the manufacturer's protocol. This served to cut the DNA at specified restriction sites. The mixture, with a total reaction volume of 40 microliters, was briefly spun down, then incubated in a 37°C water bath for 12 hours.

3.2.6 Ethyl Alcohol Precipitation and T4 DNA Ligation

Four microliters of 3M sodium acetate, pH 5.2, and two volumes of ice cold 100% ethyl alcohol was added to each tube containing a restriction digest. Following mixing by inversion, the tubes were incubated at -20°C for 12 hours to concentrate the DNA from solution. Next, the mixtures were centrifuged using the Eppendorf centrifuge 5417R (Hauppauge, New York) operating at 14,000 rpm for 15 minutes at 4°C. Supernatants were carefully removed and the pellets were washed twice with 70% ethyl alcohol to remove salts and other impurities. For each wash, the tubes were centrifuged for 15 minutes and 10 minutes at 13,400 rpm. Once all supernatants were removed, the pellets were vacuum dried for 10 minutes using the Labconco CentriVap DNA concentrator (Kansas City, Missouri). The pellets were dissolved in 8 microliters of nuclease free water and left to incubate for 25 minutes on ice.

One microliter of 10X T4 DNA Ligation Buffer and T4 Ligase (New England BioLabs; Ipswich, Massachusetts) was added to the each tube in order to join the restriction endonuclease generated fragments. The resulting 10 microliter reaction was gently mixed and left to incubate at room temperature (23-25°C) for 16 hours.

3.2.7 Long Range Polymerase Chain Reaction

Long range PCR (Qiagen; Valencia, California) was carried out per manufacturer's protocol, employing primers LBa1 and RB1(Appendix E), in order to amplify the regions flanking the T-DNA insertion site. The total reaction volume of 25 microliters was placed in the MJ Mini Personal Thermocycler (Bio-Rad) utilizing the following program: initial activation at 93°C for 3 minutes, denaturation at 93°C for 15 seconds, annealing at 54°C for 30 seconds, extension at 68°C for 8 minutes, holding at 4°C indefinitely for a total of 35 cycles.

To resolve the resulting PCR products, gel electrophoresis was carried out. One hundred milliliters of 1X TAE (0.023 M Tris, 0.017 M Tris-Acetate, 0.001 M EDTA) was added to a round bottom flask containing two 500 milligram agarose tablets. This 1% gel solution was constantly stirred until the tablets dissolved and weighed. The flask was boiled for 4 minutes in the microwave and swirled periodically to clear up any withstanding crystals. The solution was weighed again and any evaporated water was added back to the flask. The flask was left to stir on a stirring plate until cool to the touch. The agarose suspension was poured into the casting tray assembled within the Horizon 11·14 Life Technologies gel box (Grand Island, New York). Once solid, the gel was completely submerged in 1X TAE.

On a strip of parafilm (West Chester, Pennsylvania), one microliter of 6X EZ Vision dye was added to five microliters of each PCR product. For the molecular weight lane, two microliters of 1kb ladder and three microliters of nuclease-free water were combined with one microliter of the dye. Each sample was loaded into the gel and run at 93 volts for two hours. The presence of PCR products was visualized using UltraCam

Digital Imaging (Vexcel; Boulder, Colorado) and the Foto/PrepI UV Transilluminator (Fotodyne; Hartland, Wisconsin) and photographed with the PowerShot A620 (Canon; Lake Success, New York).

3.2.8 Gel Extraction and DNA Sequencing

PCR products of interest were excised with a clean scalpel and purified using the QIAquick Gel Extraction kit following the vendor's protocol. The concentration of the collected DNA was determined by spectrophotometer analysis. The Beckman Coulter GenomeLab Dye Terminator Cycle Sequencing with Quick Start kit was employed using primers LBb1 and RB2 (Appendix E) as per manufacturer's instructions to determine the order of the nucleotide bases flanking the T-DNA insertion site. The recovered sequences were assembled using Vector NTI Software (Invitrogen; Grand Island,New York) and analyzed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to reveal possible sequence homologies in *P. marneffei* and other organisms.

3.2.9 Slide Cultures

To examine the morphology of *P. marneffei* strains under different growth and temporal conditions, slide cultures were conducted in a manner similar to that described by Harris (72). Slide cultures are used to immortalize the structures of an organism without disturbing them. Petri dishes containing BHIA, MEA, and PDA were made. Each plate was cut into multiple, small squares with a flame sterilized scalpel. In the same manner, sterile forceps were used to place a glass coverslip upon the surface of a

petri dish containing water agar. A small square from each substrate was placed on top of the coverslip. A cotton swab dipped in sterile distilled deionized water was used to swab an isolated colony of the either the wild type or mutant. The sides of the substrates were inoculated with the fungal strain, and another coverslip was placed on top to sandwich the agar square. The petri lid was replaced to cover the entire dish. Each strain had one plate and was incubated at 25°C and 37°C for one week.

To observe the morphology of each strain using microscopy, a small drop of Lactophenol Cotton Blue with Polyvinyl Alcohol (LPCB-PVA; lactic acid, phenol, PVA, aniline blue) was added to the center of a labeled microscope slide (73). The top coverslip from each seven day slide culture was removed with sterile forceps and positioned on top of the drop of dye. The agar square was discarded, and the bottom coverslip was handled in the same manner. Once the dye completely covered the slip, the microscope slide was viewed with a differential interference contrast (DIC) microscope (Olympus; Center Valley, Pennsylvania) and the SPOT BASIC 4.6 software program (Diagnostic Instruments; Sterling Heights, Michigan) was used to take photomicrographs.

3.2.10 Southern Blot

To determine the T-DNA copy number present within the mutant, Southern Blot analysis was performed. The probe was prepared amplifying the *ble*R gene that resides within the T-DNA region of plasmid UPRS0 (Appendix A) using primers Gpda-F and Cycl-R (Appendix E). The PCR product was run on a 1% gel, and the 752 base pair band was excised and gel purified. Once the concentration of the eluate was recorded, it was stored at -20°C.

Wild type and mutant DNA were digested for 12 hours with enzymes *Nde*I or *Kpn*I. The digests as well as intact genomic DNA were run on a 1% gel for 3 hours and 30 minutes at 60 volts. According to the protocol provided by the Whatman® Turboblotter™ System (GE Healthcare Biosciences; Pittsburgh, Pennsylvania), the gel was denatured and transferred to a Nytran® SuPerCharge nylon membrane. The membrane was neutralized and cross-linked with ultraviolet light (254 nm). Next, the probe was labeled with horseradish peroxidase and hybridized to the membrane using the Amersham ECL Direct Labeling and Detection System, according to protocol B of the vendor's manual. To detect the generated signal, the membrane was then added to an Amersham Hypercassette and taken to the darkroom. An autoradiography film was added to the cassette and allowed to incubate with the membrane for 1 minute. It was developed for 30 seconds (Kodak; Rochester, New York), rinsed with distilled water for 10 seconds, fixed for 15 minutes, rinsed again for 30 seconds in distilled water and dried. The detection process was repeated once again, this time exposing the film for approximately 5 hours.

3.2.11 Colony Radial Growth Assay

Radial growth assays were implemented to assess the rate at which the mutant grows in comparison to the wild type on various carbon sources. Seven-day-old conidia from each isolate were collected, counted, and adjusted to 1×10^6 conidia per milliliter as previously mentioned. Three microliters of fungal suspensions was added to the center of petri dishes containing MEA, PDA, and SDA media in a balanced 3 x 2 (medium x fungal strain) factorial design, with 5 petri dishes in each cell for a total *n* of 30. All

plates were left to incubate at 25°C, with colony diameter measurements taken with a ruler every 24 hours for one week. The collected data were then subjected to a two-way repeated measures ANOVA, with day of measurement as the within-subjects repeated measure (PASW Statistics 18).

A subsequent assay was put into place using minimal media (MM, Appendix F). The experiment was carried out utilizing the aforementioned protocol and conditions with the following adjustments. The MM plates were supplemented with 0.05%, 1%, 2% glucose. Three microliters of fungal suspensions was added to the center of petri dishes in a balanced 3x 2 (medium x strain) factorial design, with 3 petri dishes in each cell for a total *n* of 18. All plates were left to incubate at 25°C, with colony diameter measurements taken with a ruler every 24 hours for ten days.

3.2.12 Drop Dilution Assay

Beginning with 1 x 10^7 conidia per milliliter, ten-fold serial dilutions of the wild type and mutant suspensions were prepared to investigate the sensitivity of the mutant to different stressors. Five microliters of each dilution was added to a petri dishes containing PDA supplemented with varied concentrations of sodium dodecyl sulfate (SDS), a detergent known to disrupt the cell membrane (74,75) and Congo Red, a dye identified to perturb the cell wall and affect chitin and β(1,3)-glucan assembly (76, 77). The dishes were left to incubate 25°C and 37°C. The heat shock response of the wild type and mutant was also evaluated. This was accomplished by incubating each strain at 39°C on PDA only. After 4 days, the presence of colony growth for all stress conditions was recorded.

3.2.13 Complimentary DNA Synthesis and Expression Studies

Aliquots (~400 µl) of wild type and mutant fungal cells grown at 25°C and 37°C for 48 hours were retrieved from -20°C storage and thawed on ice. In order to release the contents of the cells, biomass from each strain was added to 2 ml tube containing 0.5 mm glass beads and disrupted using the Mini-Beadbeater (BioSpec Products; Bartlesville, Oklahoma). Total RNA was isolated using the RNeasy Mini Kit per manufacturer's protocol. DNaseI was applied to each sample with the purpose of eliminating all traces of DNA. The RNA was amplified using primers for the chitin synthase B (*chsB;* Appendix E*)* gene to check for DNA contamination. The products, which were run on a 1% gel, presented no banding while the lane containing the positive control possessed a band ~500 base pairs (data not shown), thereby indicating the absence of DNA in the RNA samples. Reverse transcription and amplification of 100 nanograms of DNaseI-treated RNA were carried out simultaneously using the Qiagen® OneStep RT-PCR Kit. Following the accompanying vendor's handout, this experiment was performed to examine whether a transcript was being produced by the mutant in mold and yeast phases. Ufdb5 RT-F/Ufdb5 RT-R and Ufdb3 RT-F/Ufdb3 RT-R (Appendix E) were designed to prime the regions before and after the deletion, respectively. Primers for tubulin beta (Appendix E), a housekeeping gene in *P. marneffei*, were used as a positive control to ensure cDNA synthesis properly occurred.

CHAPTER 4 RESULTS

4.1 Morphological Assessment

Penicillium marneffei F4 (wild type) and I189 (mutant) strains were grown at 25°C on malt extract agar (MEA) for 7 days. At day four, the wild type displayed off-white to fawn colored conidia that were fluffy in appearance (Figure 2). As the culture continued to mature, the mycelia became thick and numerous. The mutant produced vibrant yellow spores after 4 days of growth that gradually faded as they fully developed on day 7.

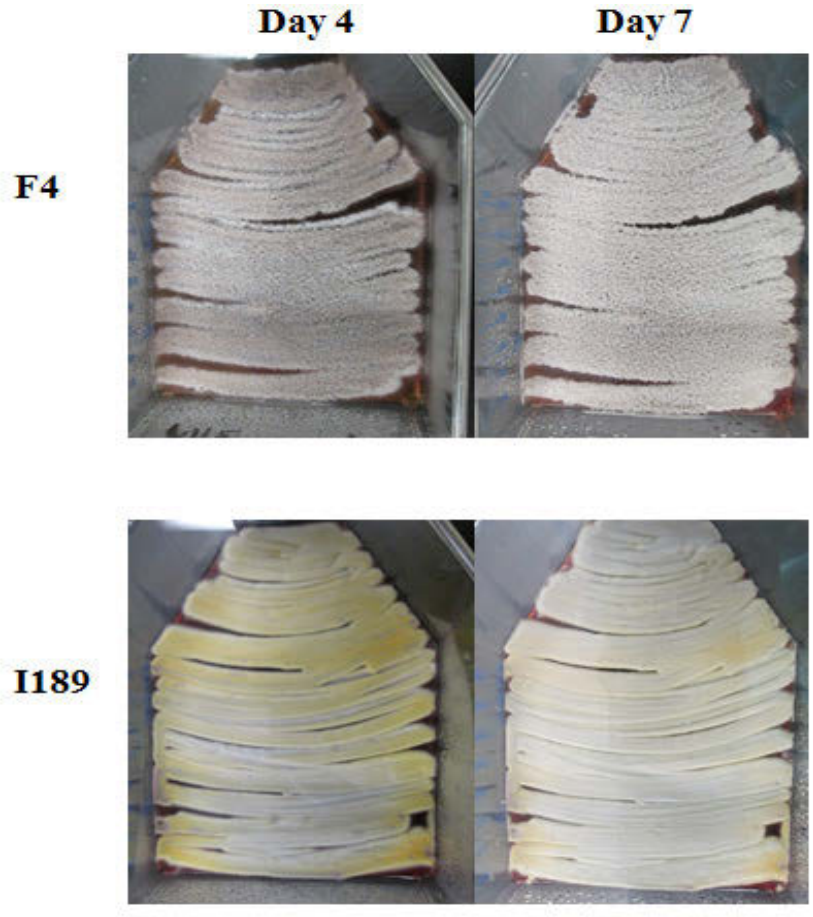

Figure 2 Growth pattern of F4 (wild type) and I189 (mutant) at 25 °C on day 4 and day 7. Each strain was cultured on malt extract agar for 7 days.

At 37°C, each isolate was grown on brain heart infusion agar (BHIA) for 7 days. The wild type produced a plethora of light brown yeast colonies and diffused a brown to amber colored pigment into the agar (Figure 3). The mutant generated numerous yeast colonies however, little to no pigment was present. Thus, the mutant differs phenotypically from the wild type in pigment production at both 25°C and 37°C.

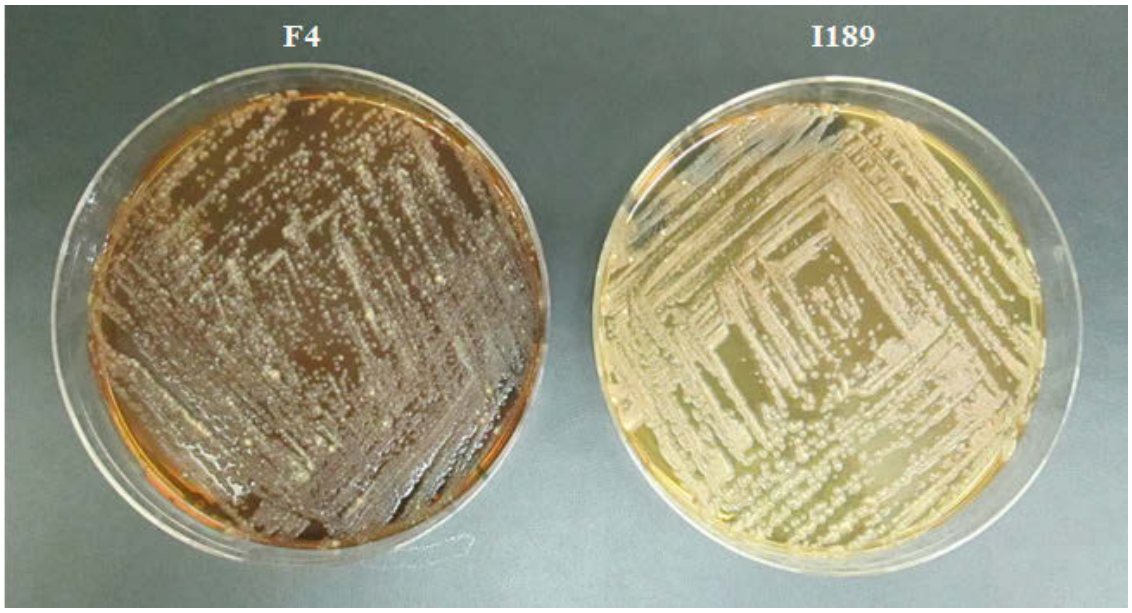

Figure 3 Growth pattern of F4 (wild type) and I189 (mutant) at 37 °C. Each strain was cultured on brain heart infusion agar for 7 days.

4.2 Slide Cultures

Slide cultures were performed to evaluate the orientation of the wild type (F4) and mutant's (I189) conidiophores and other structures on different growth media. At 25°C, the phialides of F4 produced long chains of intact conidia on PDA and MEA. On BHIA, well-formed hyphae were generated and defined cell walls were present (Figure 4A). At the same temperature, the conidiophores of I189 created nicely formed conidia on PDA and MEA.

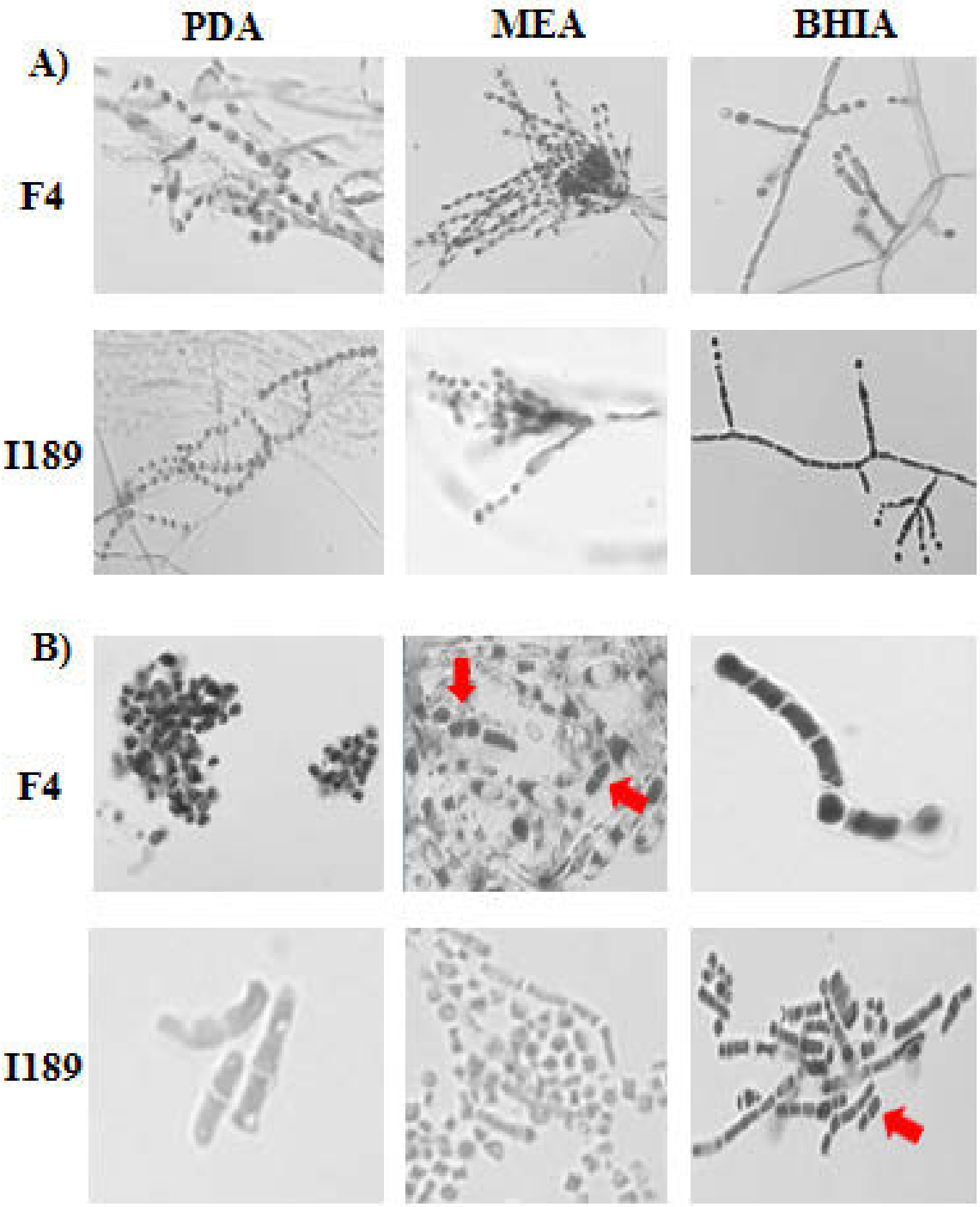

Figure 4 Differential interference contrast photomicrographs of F4 (wild type) and I189 (mutant) slide cultures. The isolates were grown on potato dextrose agar (PDA), malt extract agar (MEA), and brain heart infusion agar (BHIA) for seven days at (A) 25°C or (B) 37°C. The red arrows indicate double septate walls of the yeast cells.

Distinctly segmented cells were seen on BHIA, similar to that of the wild type.

An aggregation of F4 yeast cells grown at 37°C were observed on PDA and MEA, with a short chain of arthroconidia on BHIA (Figure 4B). Phase transitioned I189 yeast cells were observed on PDA, MEA, and BHIA, along with the presence of arthroconidia. Microscopically, there were no major differences in formation and maturation of the mutant's structures when compared to the wild type.

4.3 T-DNA Verification

Wild type and mutant strains of *P. marneffei* were grown at 37°C on BHIA supplemented with 4 μg/ml of bleomycin in order to confirm T-DNA integration. After 3 days, many yeast colonies were present on the petri plate streaked with I189 (Figure 5). The plate inoculated with F4 contained no colonies, and served as a negative control. Hence, these results strongly suggest that I189 contains the *ble*R gene as a result of ATMT.

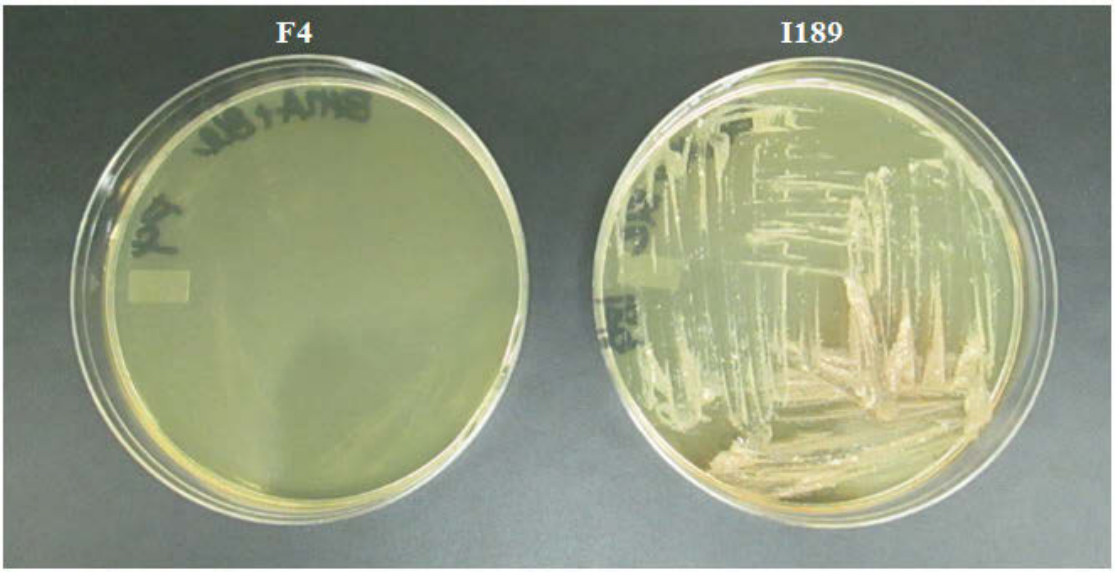

Figure 5 Confirmation of T-DNA integration. Wild type and mutant DNA were streaked on brain heart infusion agar (BHIA) supplemented with 4 µg/ml of bleomycin for 3 days at 37°C. F4 (left) served as the negative control.

4.4 Inverse PCR and DNA Sequencing

Genomic DNA from F4 and I189 were digested, self-ligated, and amplified with primers designed to amplify the regions flanking the T-DNA insertion site using inverse PCR. Lane 1 contained a one kilobase molecular weight ladder (Figure 6). Lanes 2-5 were loaded with F4 that was digested with enzymes *Nde*I, *Xho*I, *Bam*HI, and *Xba*I, respectively. Lanes 6-9 were loaded with I189 that was digested with the aforementioned enzymes. No PCR products were generated from the wild type. However, an amplification product (~2.6 kb) was observed from the mutant strain DNA digested with *Nde*I. Excision and subsequent sequence analysis of this PCR product revealed homology to a mRNA encoding an ubiquitin fusion degradation protein (UfdB; NCBI Reference Sequence: XP_002144358.1) (Figure 7). Analysis of the deduced amino acid sequence of UfdB from I189 revealed that the integration of the T-DNA generated a deletion of three nucleotides. The absence of these nucleotides occurred downstream

from the ubiqutin elongating factor core coding sequence and upstream from the RING (Really Interesting New Gene) finger domain coding sequence.

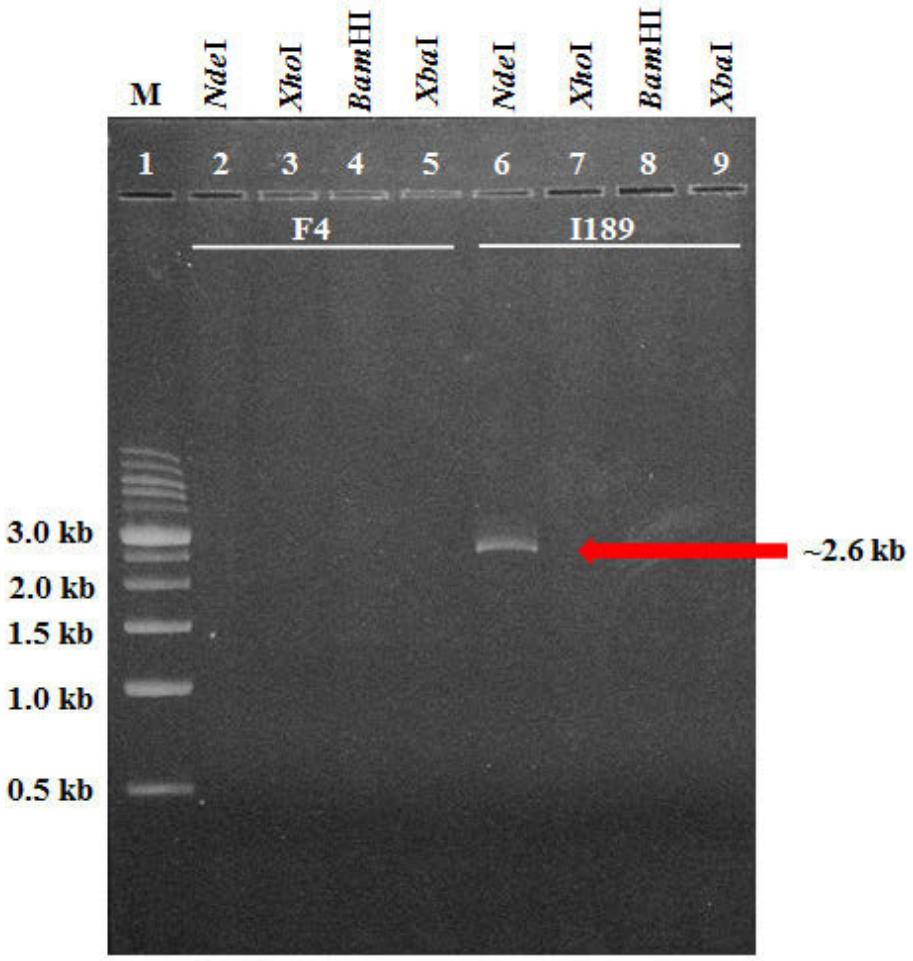

Figure 6 Inverse PCR products resulting from T-DNA integration. Amplification products were resolved on a 1% agarose gel. Lane 1 contained the 1 kilobase molecular weight ladder. Lanes 2-5 contained F4 (wild type) products and lanes 6-9 possessed I189 (mutant) products. Mutant DNA previously digested with *Nde*I resulted in a band approximately 2.6 kilobases (red arrow).

4.5 Southern Blot Analysis

After digestion with either *Nde*I or *Kpn*I, wild type and mutant genomic DNA were subjected to Southern Blot analysis to ensure that only one T-DNA copy integrated within the genome of I189. The first lane contained a one kilobase ladder (Figure 8).

Lanes 2-5 contained either F4 or I189 digested with either *Nde*I or *Kpn*I. Lanes 6 and 7 were loaded with wild type and mutant genomic DNA, respectively.

Penicillium marneffei ATCC 18224 ubiquitin fusion degradation protein UfdB, putative, mRNA

Sequence ID: ref|XM_002144322.1| Length: 3192 Number of Matches: 1

Range 1: 2074 to 2909 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1365 bits(739)	0.0	815/850(96%)	15/850(1%)	Plus/Minus

```
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Query  743   AAGTCGAGATTCTCATTTGCCTGATCGGAAAGTTGATTTCTATAAGTTGGGGTTGCCTCC  802
             |||||||||||||||||| |||||||||||||||||||||||||||| |||| |||
Sbjct  2309  AAGTCGAGATTCTCATTTGCTTGATCGGAAAGTTGATTTCTATAAGTTGGG-TTGC-TCC  2252
```

Figure 7 Sequence analysis using BLAST. The DNA product was discovered to be an ubiquitin fusion degradation protein (UfdB) mRNA.

The labeled probe was directed toward the *ble*R marker within the T-DNA insert. There were no hybridization signals in both F4 lanes. Single 6.5 and 6 kb hybridization signals appeared in lanes 4 and 5, respectively, containing I189. This strongly suggested that only one T-DNA copy integrated the genome.

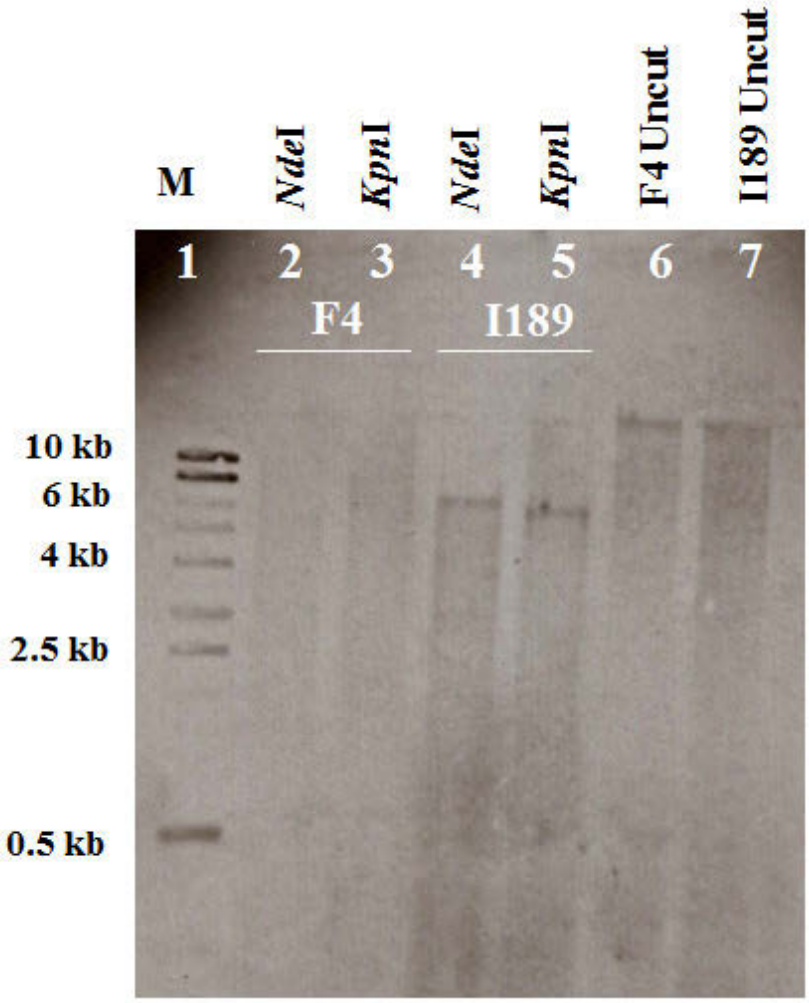


Figure 8 Southern Blot analysis of F4 (wild type) and I189 (mutant). Lane 1 possessed the 1 kilobase molecular weight ladder. Serving as the negative control, F4 displayed no hybridization signals in lanes 2 and 3. Single 6.5 and 6 kilobase bands appeared in lanes 4 and 5 which contained I189 digested DNA. Uncut wild type DNA was added to lane 6 and uncut mutant DNA was added to lane 7.

4.6 Expression Studies

Reverse transcription-PCR revealed that the mutant was able to produce mRNA transcripts upstream and downstream of the T-DNA insertion site (Figure 9). This occurred in both the mold (25°C) and yeast (37°C) phases. The upstream PCR products were ~311 base pairs and the downstream PCR products were ~199 base pairs. The bands were of the same size as the wild type. Housekeeping gene tubulin beta was used as a positive control and resulted in bands ~200 base pairs in both strains at each temperature. These results suggest that the protein coding regions upstream and downstream of the T-DNA insertion were transcribed. This assay, however, does not provide definitive information regarding the function of the translated product.

4.7 Radial Growth

To gain an understanding of how the mutant germinates in response to nutrients supplied by different growth media, a colony radial growth assay was implemented. One-week-old spore suspensions were added to petri dishes containing MEA (Figure 10A), PDA (Figure 10B) and SDA (Figure 10C). After seven days, the mutant produced yellow pigmented conidia, while the wild type produced white conidia on MEA and PDA. On SDA, the wild type produced pink/red pigmented conidia while the mutant generated faint yellow conidia. ANOVA results demonstrate a strong interaction ($p < 0.05$; Appendix J) between the strains and among the media as they matured (Figure 11). Both strains grew at the same rate on all media on days 2 and 3 but quickly diverged on day 4. On SDA, the wild type grew exceptionally well, while the mutant lagged behind

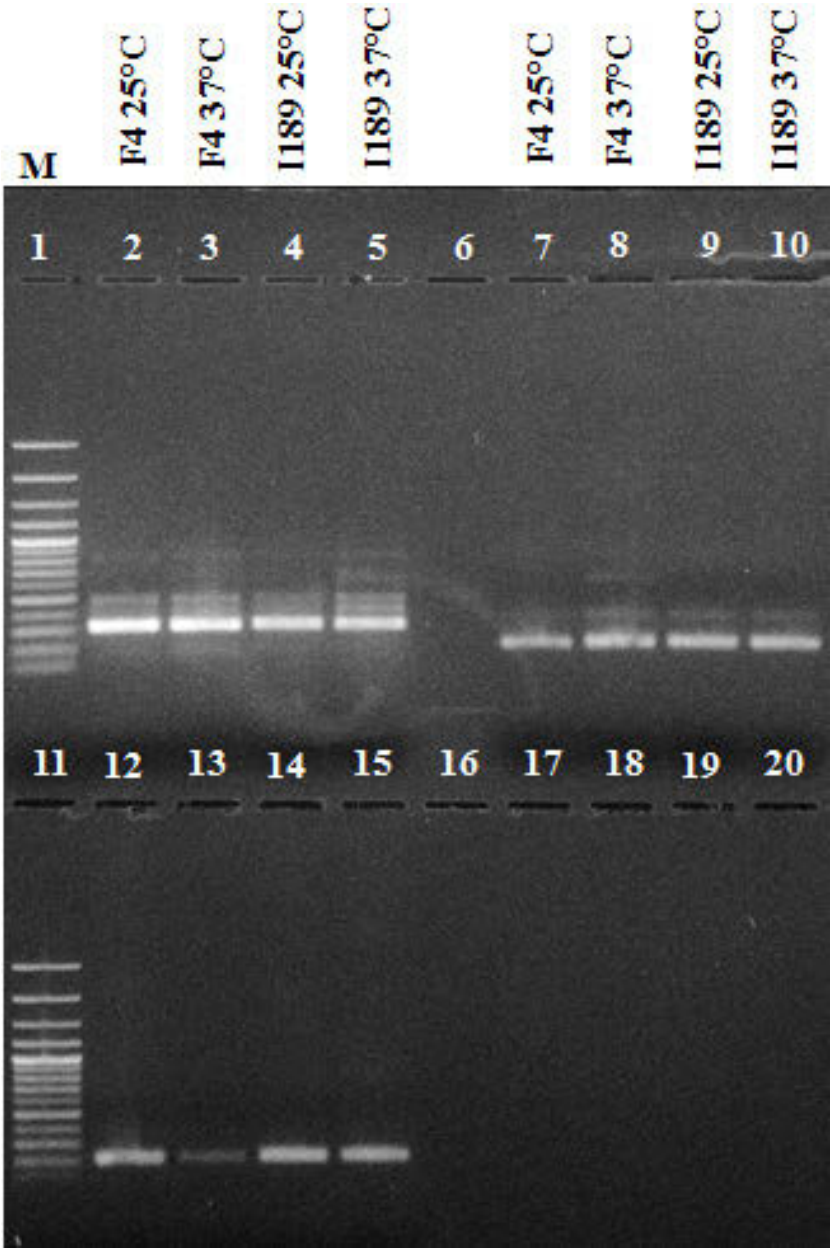


Figure 9 Reverse transcription of F4 (wild type) and I189 (mutant). Lanes 1 and 11 contained a 100 base pair molecular weight ladder. Lanes 2-5, amplified using upstream primer pair Ufdb5 RT-F/Ufdb5 RT-R, resulted in ~311 base pair bands. Lanes 7-10, amplified using downstream primer pair Ufdb3 RT-F/Ufdb3 RT-R, resulted in ~199 base pair bands. Lanes 12-15, containing F4 (25°C), F4 (37°C), I189 (25°C), and I189 (37°C), respectively, were amplified using housekeeping (tubulin beta) primer pair Btub-F/Btub-R. Each yielded a band ~207 base pairs in size. Lanes 6 and 16-20 were left empty.

after day 3. On MEA, the wild type and mutant grew at a similar rate. In contrast, on nutrient-limited PDA, the mutant's growth began to slowly decline after 4 days. However, on this same medium, the wild type's development steadily increased as it became older.

Repeated measures ANOVA revealed that proliferation for each colony grown on minimal media was influenced by the day and strain ($p < 0.05$, Figure 12), but not by the amount of glucose added (Appendix K). The wild type grew better than the mutant irrespective of the amount of glucose present. Over time, there was an apparent divergence between and tight grouping of the two strains as well. Collectively, these results suggest that I189 may be sensitive to nutrient-limiting conditions.

4.8 Drop Dilution Assay

At 25°C and 37°C, the mutant was slightly more resistant to Congo Red than the wild type (Figure 13E, F). Under SDS (Figure 13C, D) and heat conditions (Figure 13G), conidia from the mutant were able to grow and survive at the same dilutions as its wild type counterpart. These observations suggest that the nature of the mutant's interruption may not play a role in resolving perturbations affecting the cell wall and cell membrane or heat shock.

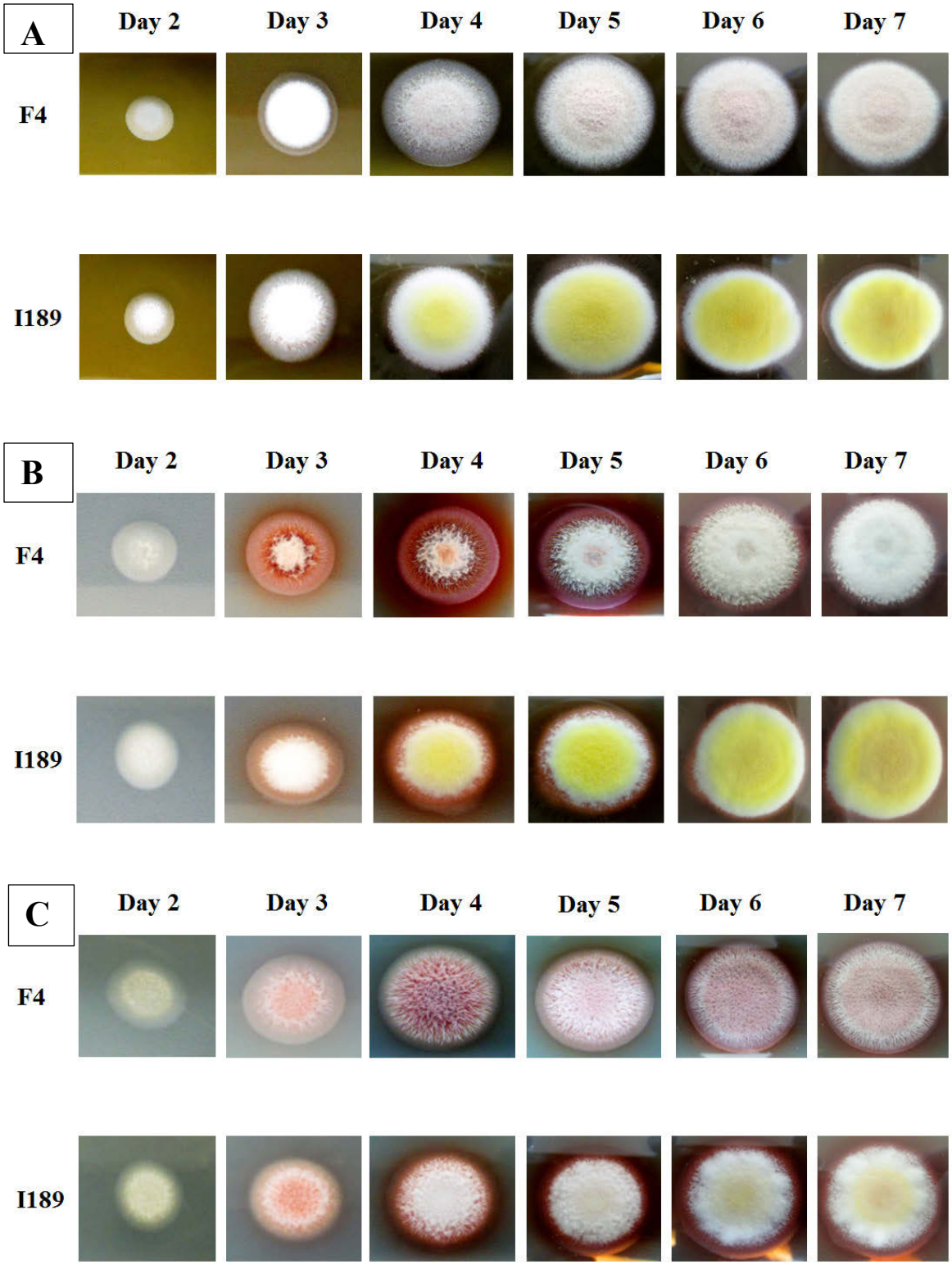


Figure 10 Colony radial growth of F4 (wild type) and I189 (mutant) on MEA, PDA, and SDA. Each strain was grown at 25°C on MEA (A), PDA (B), and SDA (C) for seven days.

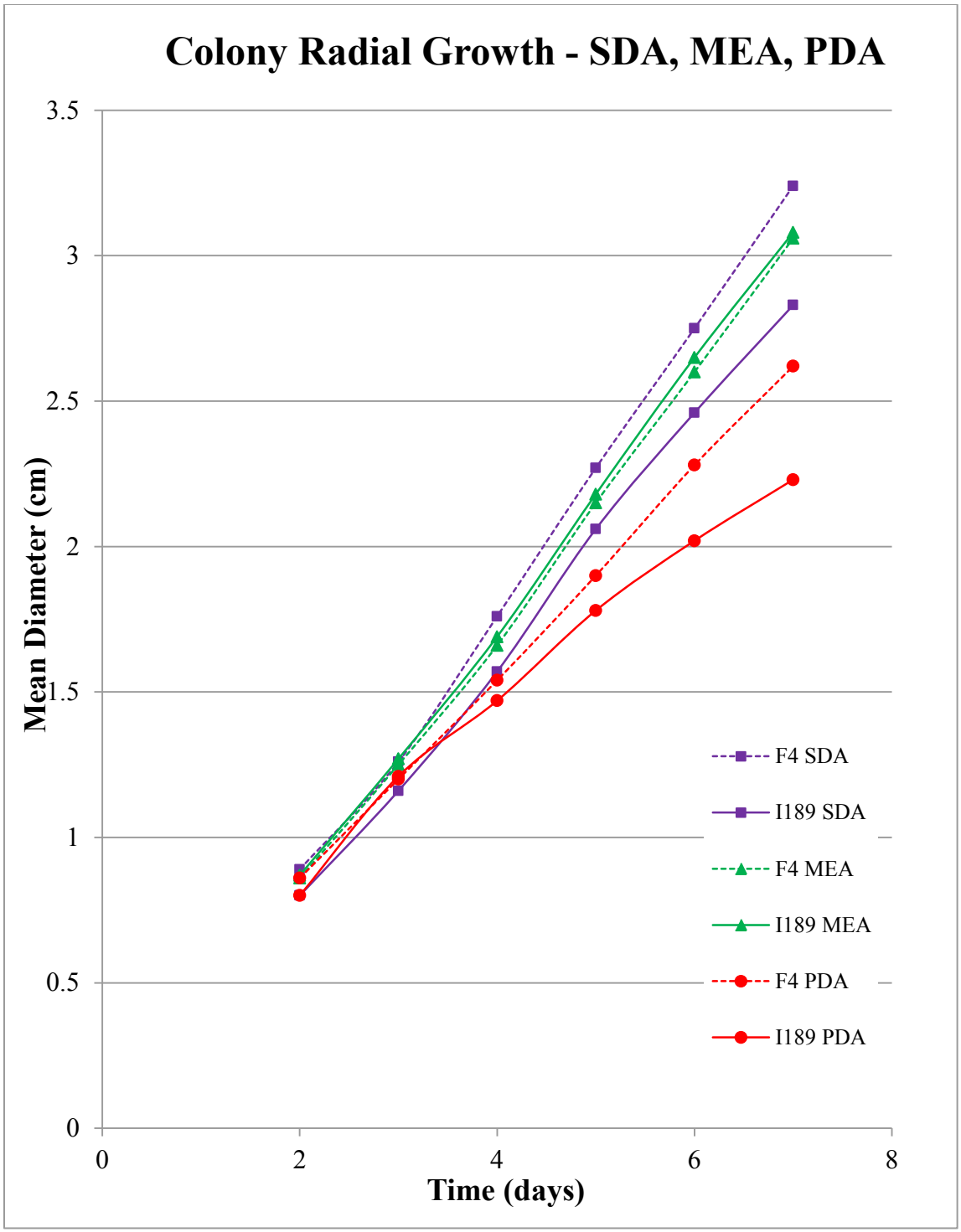


Figure 11 Time series plots of F4 (wild type) and I189 (mutant) radial growth on SDA, MEA, and PDA. The standard error for each series was less than 0.04 and is therefore not shown. F4 is indicated by dashed lines and I189 with solid lines. The media are denoted by a (■) for SDA, (▲) for MEA, and (●) for PDA.

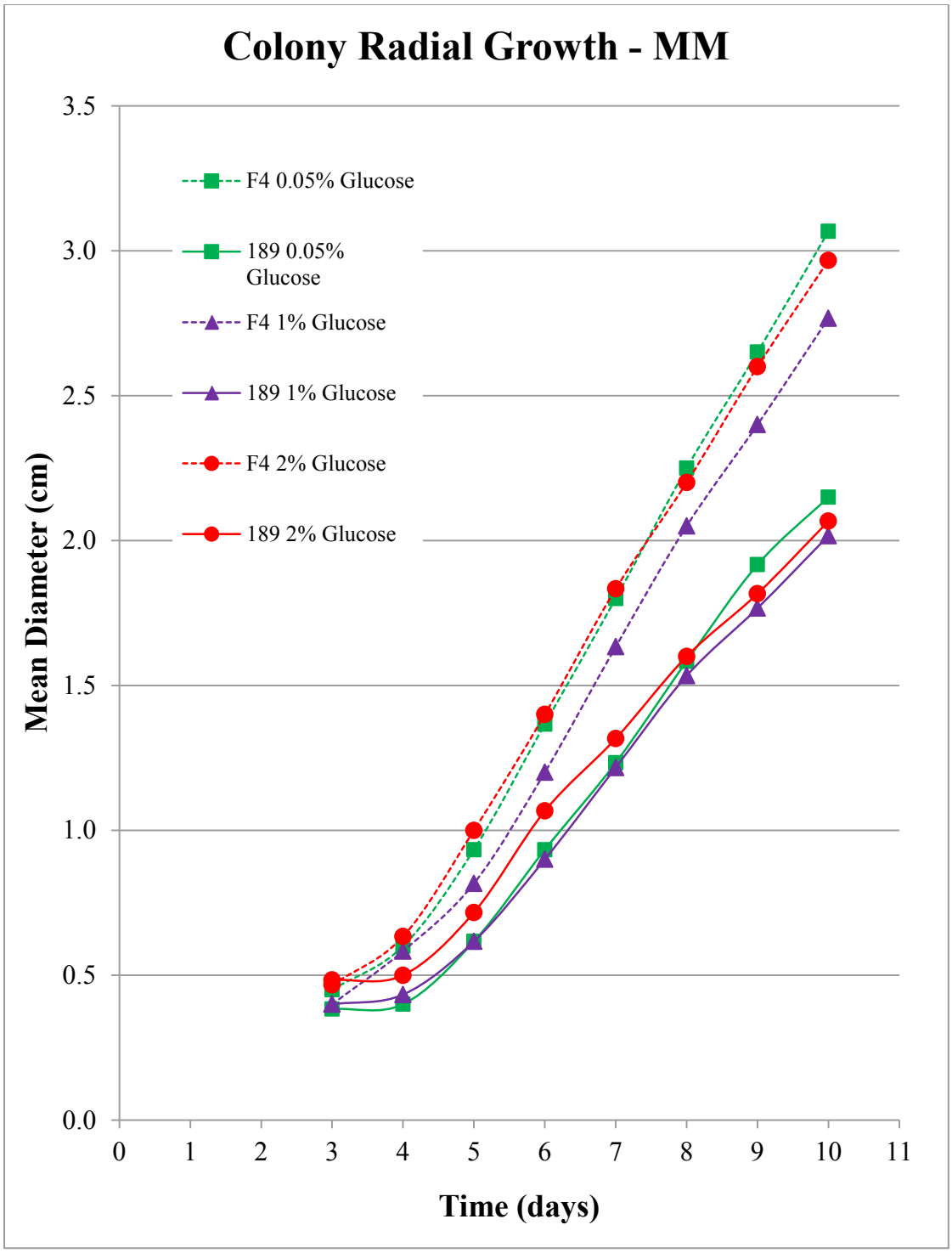


Figure 12 Time series plot of F4 (wild type) and I189 (mutant) radial growth on MM supplemented with 0.05%, 1%, and 2% glucose. The standard error for each series was less than 0.07 and is therefore not shown. F4 is indicated by dashed lines and I189 with solid lines. The amount of glucose is denoted by a (■) for 0.05%, (▲) for 1%, and (●) for 2%.

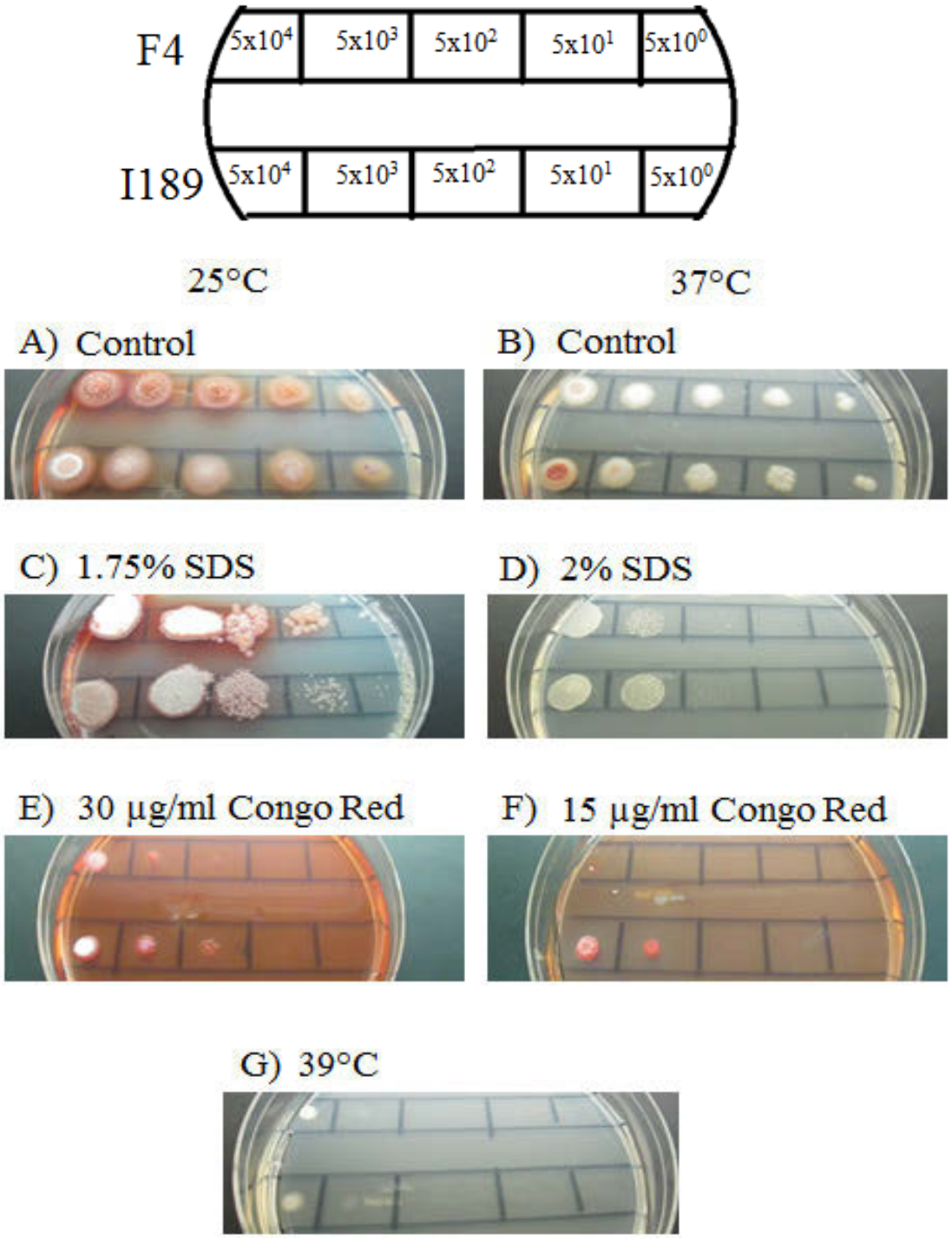


Figure 13 Drop dilution assay. Ten-fold dilutions of wild-type and mutant conidia were exposed to PDA supplemented 1.75% and 2%SDS (C, D) and 30µg/ml and 15 µg/ml Congo Red (E, F). Heat shock (G) conditions were also assessed. All plates were incubated 25°C (A, C, E), 37°C (B, D, F), or 39°C (G), and proliferation was documented after 4 days.

CHAPTER 5 DISCUSSION

This investigation sought to characterize *Penicillium marneffei* mutant I189, a transformant that produces yellow pigmented conidia at 25°C and does not release brown pigment into the agar when cultivated at 37°C. It was hypothesized that the gene responsible for these phenotypes may be involved in melanin biosynthesis and secretion. Rather, molecular analysis of I189 uncovered a T-DNA insertion of the ubiquitin fusion degradation protein (UfdB*)* open reading frame. Ubiquitin, a highly conserved polypeptide consisting of 76 amino acid residues, is involved in labeling proteins for autophagosomal, lysosomal, and proteosomal degradation (78). The proteins directed for degradation are typically rich in proline, glutamic acid, serine, and threonine. These regions possess phosphorylation sites required for the breakdown of amino acids (79, 80). The ubiquitin-proteasome system is responsible for the turnover of many cellular components, including regulatory and transcriptional elements. It also serves to remove unraveled, disassembled, and misfolded proteins (81, 82).

Ubiquitination occurs with the aid of three enzymes. Once target proteins are sent by the 26S proteasome to be degraded, the ubiquitin-activating enzyme E1 modifies ubiquitin so that its carboxy-terminal glycine residue is charged. This step calls for the hydrolysis of ATP and yields an ubiquitin adenylate. This intermediate binds to the cysteine residue of E1 through a thioester bond and is then transferred to the cysteine residue located in ubiquitin-conjugating enzyme, E2. The molecule is carried to ubiquitin-ligase enzyme E3, where an isopeptide bond is formed between the carboxy-terminal glycine residue of ubiquitin and the ε-amino group of lysine residues in the

substrate. The HECT (Homologous to E6-AP C-Terminus) or RING domain is one of two domains the E3 enzyme may possess, but both are important in recognizing the substrate to be degraded (83, 84).

Studies have shown that the few ubiquitin molecules added by E3 to the target molecule may be insufficient for degradation. At least four ubiquitin moieties must to be present on a substrate before it is acknowledged by the proteasome (85). In 1999, the multiubiquitination enzyme E4 was characterized in *Saccharomyces cerevisiae* by Koegl *et al* (86). Although not required for initial ubiquitination, this enzyme was found to discern between ubiquitin-conjugates and free standing ubiquitin and aid in the proteolysis of abnormal stress-induced proteins. Extension of the ubiquitin chain through lysine-48, one of its seven lysine residues, ensured degradation by the proteasome. These collaborators also discovered that the ubiquitin degradation pathway was not active during optimal growth conditions but became essential when cells were exposed to stress and amino acid analogs.

ufdB and its protein product UfdB have not been extensively studied in *P. marneffei.* BLAST analysis identified UfdB orthologs in *Aspergillus fumigatus* (UfdB; NCBI Reference Sequence: XP_752092.1)*, Saccharomyces cerevisiae* (UFD2; NCBI Reference Sequence: NP_010091.2)*,* and *Schizosaccharomyces pombe* (E4; NCBI Reference Sequence: NP_593630.1). All contain the ubiquitin elongating factor core, fusion degradation protein 2, and the U-box domain. Therefore, it is likely that UfdB confers the previously aforementioned functions and abilities in *P. marneffei.*

The mutant was able to grow structurally normal (i.e. conidiophores, metulae, *etc.*) as well as withstand cell wall, cell membrane, and heat stress like the wild type. It

appears that the nature of the gene interruption had no effect on hyphal morphology. However, the conidial pigment between the two strains greatly differed. The striking difference between the two specimens may merely be a consequence of subculturing the wild type for many generations and it aging, as it is typically olive green. Alternatively, the disruption of *ufdB* may have led to the improper turnover of polyketide synthase (pks) gene 11 and 12. Polyketides are secondary organic compounds released by microorganisms. *pks* 11 and *pks* 12 are responsible for the synthesis of mitorubrinic acid and mitorubrinol. It has been documented that the yellow pigment produced in the mold phase of *P. marneffei* is composed of mitorubrinic acid and mitorubrinol (87, 88). Delayed or inhibited degradation of these metabolites may have resulted in an increase of its abundance and observed phenotype. Altered protein turnover may also be responsible for the absence of brown pigment in the agar of the yeast form of the mutant, but further investigation is needed to address this notion.

It was only on media supplemented with malt extract that the mutant grew comparably to the wild type. Conversely, when cultured on minimal media supplemented with glucose, the mutant grew significantly slower than the wild type. It was also noted that the amount of glucose within each strain was non-significant in radial proliferation. The poor growth exhibited by the mutant may be caused by the amino acid content available from the growth medium for it to use. Malt extract, a product of malting barley grain, is protein and amino acid rich (89). The malt extract agar (OXOID) utilized in this investigation contained 3% malt extract, and was additionally supplemented with 0.5% mycological peptone. Peptones are also amino acid rich. To offset the lack of amino acids following insufficient tagging and degradation, it is

possible that the mutant was able to make use of the amino acid reserves presented by this growth medium and proliferate at rate similar to that of the wild type. Sabouraud's dextrose agar (BD Difco) contained a total of 1% enzymatic digest of casein and enzymatic digest of animal tissue. This amount appeared to be limiting as the mutant failed to proliferate as quickly as the wild type. Potato dextrose agar (BD Difco) and minimal media (Appendix F) contained little to no protein content, and the radial growth rate of the mutant on these media was the poorest. It is probable that the mutant may have been forced to synthesize new amino acids as a result of being unable to attain them from proteolysis or the growth media. This extra amino acid synthesis step may have caused the growth rate of the mutant to decrease significantly in comparison to the wild type, as this process is time and energy dependent. Proper protein turnover provides the building blocks required to create new polypeptides, and disturbances in protein degradation can affect growth of the organism as well as other cellular processes (90).

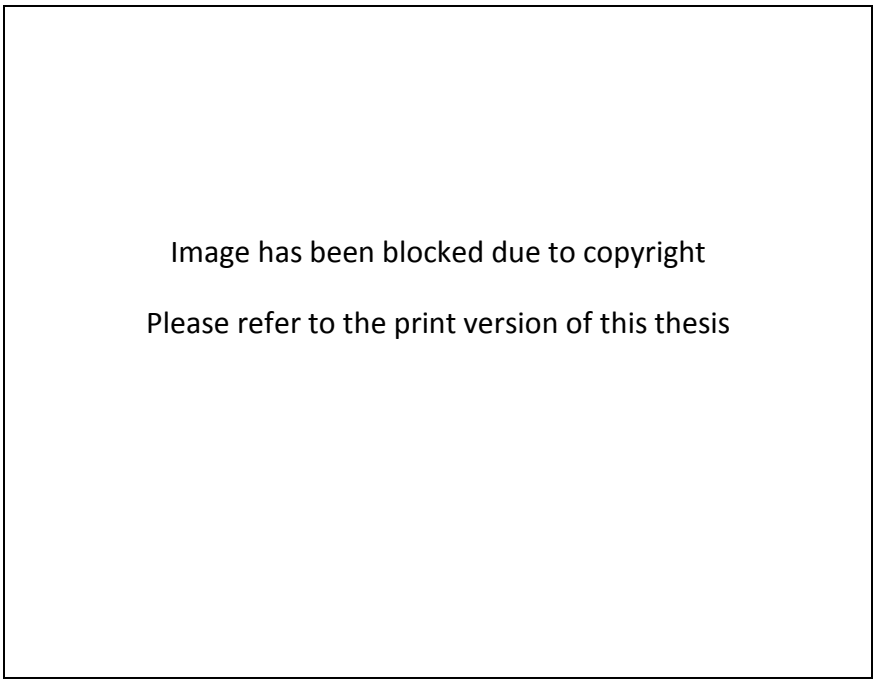
For future endeavors, the *ufdB* gene should be transformed back into the mutant in order to restore the phenotype to that of the wild type in a complementation assay. Molecular results displayed that the mutant produced an mRNA transcript upstream and downstream of the interruption. Typically, mRNA abundance does not correlate to protein expression (91). In fact, the translated protein product may not function correctly and/or possess an adverse phenotype. A Western blot, or immunoblot, should be carried out using antibodies toward UfdB to detect for its presence. Also, single-molecule fluorescence could be used to detect and identify any possible misfolded, native state proteins (92). The colony radial growth rate of the wild type and mutant should be evaluated after being plated on minimal media supplemented with varying quantities of

amino acids. The goal would be to assess whether amino acid limitation is responsible for the growth rate phenotype. Finally, the virulence of this mutant should be assessed. The best model would be the wax moth *Galleria mellonella*, an insect found naturally in beehives. Containing six types of phagocytic cells, it possesses an innate immune system similar to that of humans (93, 94). *G. mellonella* looks to be a novel *in vivo* model to investigate the manner by which *P. marneffei* causes disease.

P. marneffei is infamous for causing disseminated infection within persons suffering from HIV/AIDS. Its thermal dimorphism, or ability to thrive as a mold or yeast based on temperature signals, has fascinated investigators far and wide as the method by which it occurs still remains unclear. Although the genomes of *P. marneffei* strains ATCC 18224 and PM1were recently sequenced and released (95, 96), a large portion of their genes have yet to be characterized. Kummasook *et al.* (67) utilized the technique of *Agrobacterium tumefaciens*-mediated transformation in *P. marneffei* to identify novel protein coding sequences possibly responsible for phase transition. Further investigation of mutant I189 as well as other transformants generated by these collaborators should be to be done in order to gain knowledge about the mechanisms of this significant, pestilent fungus.

CHAPTER 6 APPENDICES

Appendix A: Map of pUPRS0



ble.- bleomycin resistance gene from *Streptoalloteichus hindustanus*

*Km*R.- kanamycin resistance gene

*Amp*R.- Ampicillin resistance gene

P*gpdA*.- Glyceraldehyde 3´- phosphate dehydrogenase gene promoter from *Aspergillus nidulans*

T*CYC*1.- Cytochrome 1 oxidase transcripcional terminator

LB and RB.- Left and Right borders of the transference DNA (T-DNA)

P*nos*.- nitric oxide synthase gene promoter

*npt*II.- neomycin phosphotransferase II gene

T*nos*.- nitric oxide synthase transcriptional terminator

(reproduced from Cardoza *et al.*, 2006, with permission from the authors (68; Appendix I)

Appendix B: T-DNA nucleotide sequence

Left border repeat

TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATT
GCGGACGTTTTTAATGTACTGGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAG
CTGATTGCCCTTCACCGCCTGGCCCTGAGAG**AGTTGCAGCAAGCGGTCCACGC(LB b1)**TGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTCCGAAATCGGCAAA
ATCCCTTATAAATCAAAAGAATAGCCCGAGATAGGGTTGAGTGTTGTTCCAGTTTGG
AACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGT
CTATCAGGG**CGATGGCCCACTACGTGAACCA(Lba1)**TCACCCAAATCAAGTTTTTTG
GGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAG
AGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAA
GGAGCGGGCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCT
CTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGG
TAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGC
TCGGTA**CCC**CTCTGTACAGTGACCGGTGACTCTTTCTGGCATGCGGAGAGACGGACG
GACGCAGAGAGAAGGGCTGAGTAATAAGCCACTGGCCAGACAGCTCTGGCGGCTCT
GAGGTGCAGTGGATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAGTGGA
AAGGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAG
CTTCATCGAATCACCGGCAGTAAGCGAAGGAGAATGTGAAGCCAGGGGTGTATAGC
CGTCGGCGAAATAGCATGCCATTAACCTAGGTACAGAAGTCCAATTGCTTCCGATCT
GGTAAAAGATTCACGAGATAGTACCTTCTCCGAAGTAGGTAGAGCGAGTACCCGGC
GCGTAAGCTCCCTAATTGGCCCATCCGGCATCTGTAGGGCGTCCAAATATCGTGCCT
CTCCTGCTTTGCCCGGTGTATGAAACCGGAAAGGCCGCTCAGGAGCTGGCCAGCGGC
GCAGACCGGGAACACAAGCTGGCAGTCGACCCATCCGGTGCTCTGCACTCGACCTGC
TGAGGTCCCTCAGTCCCTGGTAGGCAGCTTTGCCCCGTCTGTCCGCCCGGTGTGTCGG
CGGGGTTGA**CAAGGTCGTTGCGTCAGTC(**Gpda-F)
CAACATTTGTTGCCATATTTTCCTGCTCTCCCCACCAGCTGCTCTTTTCTTTTCTCTTTC
TTTTCCCATCTTCAGTATATTCATCTTCCCATCCAAGAACCTTTATTTCCCCTAAGTAA
GTACTTTGCTACATCCATACTCCATCCTTCCCATCCCTTATTCCTTTGAACCTTTCAGT
TCGAGCTTTCCCACTTCATCGCAGCTTGACTAACAGCTACCCCGCTTGAGCAGACATC
ACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGA
GCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGAC
TTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAG
GTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTAC
GCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATG
ACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGG
CAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTGACCGACGCCGACCAACACCG
CCGGTCCGACGCGGCCCGACGGGTCCGAGGCCTCGGAGATCCGTCCCCCTTTTCCTTT
GTCGATATCATGTAATTAGTTAT**GTCACGCTTACATTCACGC(**Cyc1-R)
CCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAG
GTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAAT
TTTTCTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACTGAAAACCTTG
CTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTTGGCGTAATCATG
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGA
GCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATT
AATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCCAAAGACAAAA
GGGCGACATTCAACCGATTGAGGGAGGGAAGGTAAATATTGACGGAAATTATTCATT

AAAGGTGAATTATCACCGTCACCGACTTGAGCCATTTGGGAATTAGAGCCAGCAAAA
TCACCAGTAGCACCATTACCATTAGCAAGGCCGGAAACGTCACCAATGAAACCATCG
ATAGCAGCACCGTAATCAGTAGCGACAGAATCAAGTTTGCCTTTAGCGTCAGACTGT
AGCGCGTTTTCATCGGCATTTTCGGTCATAGCCCCCTTATTAGCGTTTGCCATCTTTTC
ATAATCAAAATCACCGGAACCAGAGCCACCACCGGAACCGCCTCCCTCAGAGCCGC
CACCCTCAGAACCGCCACCCTCAGAGCCACCACCCTCAGAGCCGCCACCAGAACCAC
CACCAGAGCCGCCGCCAGCATTGACAGGAGGCCCGATCTAGTAACATAGATGACAC
CGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAA
ATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCAT
TACATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCG
CAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAACGAT
CGGGGATCATCCGGGTCTGTGGCGGGAACTCCACGAAAATATCCGAACGCAGCAAG
ATATCGCGGTGCATCTCGGTCTTGCCTGGGCAGTCGCCGCCGACGCCGTTGATGTGG
ACGCCGGGCCCGATCATATTGTCGCTCAGGATCGTGGCGTTGTGCTTGTCGGCCGTTG
CTGTCGTAATGATATCGGCACCTTCGACCGCCTGTTCCGCAGAGATCCCGTGGGCGA
AGAACTCCAGCATGAGATCCCCGCGCTGGAGGATCATCCAGCCGGCGTCCCGGAAA
ACGATTCCGAAGCCCAACCTTTCATAGAAGGCGGCGGTGGAATCGAAATCTCGTGAT
GGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCCCGCTCAGAAGA
ACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCG
TAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGG
GTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGG**CCACAGTCGAT**
GAATCCAGA(RB4)AAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGC
CATGGGTCACGACGAGATCATCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACA
GTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGAC
CGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGG
GCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATAC
TTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAA
TAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAAC
GCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCCTGCAGTTCATTCAGGGC
ACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGA
ACACGGCG**GCATCAGAGCAGCCGATTGTC(RB3)**TGTTGTGCCCAGTCATAGCCGAA
TAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCAT
GCGAAACGATCCAGATCCGGTGCAGATTATTTGGATTGAGAGTGAATATGAGACTCT
AATTGGATACCGAGGGGAATTTATGGAACGTCAGTGGAGCATTTTTGACAAGAAATA
TTTGC**TAGCTGATAGTGACCTTAGGCGA(RB1)**CTTTTGAACGCGCAATAATGGTT**T**
CTGACGTATGTGCTTAGCTCA(RB2)TTAAACTCCAGAAACCCGCGGCTGAGTGGCT
CCTTCAACGTTGCGGTTCTGTCAGTTCCAAACGTAAAACGGCTTGTCCCGCGTCATCG
GCGGGGGTCATAACGTGACTCCCTTAATTCTCCGCTCATGATCAGATTGTCGTTTCCC
GCCTTCAGTTTAAACTATCAGTGTTTGACAGGATATATTGGCGGGTAAAC

Right border repeat

* Primer binding sites are underlined and in red braces

**Left and Right border repeats are underlined and in blue braces

Appendix C: Inverse PCR sequences for I189

Forward-

GTGAAGGGCAATCAGCTGTTGCCCGTCTCATCTGGTGAAAAGAAAAACCACC

CCAGTACATTAAAAACGTCCGCAATGTGTTATTAAGTTGTCTAAGCGTCAATT

TGTTTACACCACAATATATCCTCTGCCTGATCATCTGCCTCCTTGGCATTCTTG

ATCCGTTTCGCTAATTCCACTAATTTAGCCAATTCTTCATCTGATTTCAGCGCT

CGTTTGCGGATGATTTCAGCTGCCTTCTCAAAATTCTGTGGTTTGTACGAACG

TCCATCCCGGGCCACGGCATAGATGAAGTTCTCCTTGTTCATCAAGTTGATAT

ACACATCGATGATCTCACTCAAAAGTCCACGGGGATTGAAGCCGTATTCTTG

CAGATTACCCACATGCAGGTTCGAGCTCTTAGGTCCCACCATCGCCTCAAGGT

TGTAGTCCAACATGTCTGCCAATCGCTGTACGATTTCGGGCATAGTGAATGAA

TCTGCTAATGCCTCTGTGAAGAGTTTGAGCATGGCTACTGTTTCGTTGGTGAG

TTGCATGTAGGATTTGGCTTGGCGTTGAGCTGCACTCAGGGCCTCTTCCTTCT

TCTTGCCGTACTGTCTGCTCCATGGTGTTTCCTTCTCGGTTGAGTAGCTCTTGC

GTATCGTGGATGGTAATAAATGCAGTAAATGCTTCATCAAGAACGAATGTCA

CGTCGTTTAAGAGAAGGTTGACAAATCGAACGAAGAAGTCGAGATTCTCATT

TGCTGGATCGGAAAGTTGATTTCTATAAGTTGGGGTTGCTCCAGATGCACCTT

GAATGAATCTGGGAAGAATTTTCGTTATTCGAAATTATTTGGAAACCCTGGGT

TCCGAAAAAAAAC

Reverse-

GATAACGAGTCAACCCGCGGCTGAGTGGCATCCTTCAACGTTGCGTGTTCTGT

CACGTTCCAAACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTCATAAC

GTGACTCCCTTAATTCTCCGCTCATGATCAGATTGTCGTTTCCCGCCTTCAGTT

TAAACTATCAGTGTTTGAGGATTTGGGAGAAATTCCAGATGATTTCCTTGGTA

TGGTTCTCTCTTTCGAGACTCGAAAAACATTTTACTAACATATAGAAGACCCC

CTGATGTACACCCTCATGGAAGACCCAGTCATCCTACCCAACTCCAAAGTCA

CGATTGACCGCGCCACAATCCGTTCTCACTTGCTGAGCGATCCACATGATCCT

TTTAACCGAGCGCCATTGAAAATTGAAGAAGTCGTACCCAGTAAGCACCTAA

TAATCCTCAAAAACGCAAAAAGTAGCATTACTGACTGATTTGCGATAGATAT

GGACCTAAAGAAACAAATTGAAGACTTCAAAGCAGAGCGCAAAGCTGCGAA

ATTGCAGTCCATGAAGAAGGATGTTATGGATACTTCGACTGGGTGATTGCAT

AGGAAAGATATGGGTCACGGTAGCATTATCATCATTATAACTAGTGAATATA

TTTTGCGCAGGGGCCGGCCGGTGAGCGCTTGCTTGATATTTGAATCTCCTTTT

GTTCTTTTCTTTTCGAGATGATTTTTGAATTGCATTGCATGTCATAGAGGTATA

ATCATATGGAAACTCAAATCAACAAGTTTGAGCTTGAGCGACATAAATGGAT

CCACAACCCGATGCAATGGAGAACATTTGAAGAAGCCCTCAAAAAGTACAA

GGACCGACTTGACCCTGGGCCCTTTGTCTCAAATACAGCCCTTCAAGGGTTTA

CTTATTTTGACACAGGTGGTGGCACGACCTCGTTTCCATTGCAGGTTCATTGA

AGAATTTGTTATTTGTATTGGATTCCTCCCCCTCGTTTTCTGGACTGGACCTTC

CCAACAGGAAGCTTTCTTGCCCCTACGGAAAAGCCTCCGAAATTTTTAGTGTT

TCCCTGAATTTTAATTGATA

Appendix D: Pairwise alignment between the reverse complement (RC) of the ubiquitin fusion degradation gene and its mRNA

```
RC              CGGTCTTTCTTTTCTAGTATACTCCCTTGGAGGAGGGCTTGCTGACTCCGAAGATGTCGG
RNA            ----------------------------------------------------ATGTCGGG
                                                                    *******

RC              AACCGCTCTCAGATGCTGATAAGGTAAGCTTGCCTTCACGGCCGGCATACGACAGTGTCG
mRNA            AACCGCTCTCAGATGCTGATAAG-----------------------------------
                ***********************

RC              ATCTTACAACTCACTTCACTCGCTCTAGATTCGCAATAAGAGGTTAGCAAAATTAGGCGG
mRNA            ----------------------------ATTCGCAATAAGAGGTTAGCAAAATTAGGCGG
                                             ********************************

RC              AACAGCAACATCACCTGCTGCCTCACCGACAGAGTCGAGCTCCACACCGCGACCGCCGTC
mRNA            AACAGCAACATCACCTGCTGCCTCACCGACAGAGTCGAGCTCCACACCGCGACCGCCGTC
                ************************************************************

RC              GAATATCCCAACTCCACAACCACAAGCTGAGCTATCGCAGGACCAGAACGATGCTAGACC
mRNA            GAATATCCCAACTCCACAACCACAAGCTGAGCTATCGCAGGACCAGAACGATGCTAGACC
                ************************************************************

RC              AGGTGAATCCGCGCAAGGCAAGAGGATAAAGATTAGCCCCTCCACAGCAACTCCCAATGT
mRNA            AGGTGAATCCGCGCAAGGCAAGAGGATAAAGATTAGCCCCTCCACAGCAACTCCCAATGT
                ************************************************************

RC              TCCATCGCCATCGTCATCGTCATCGCCAGCGCCAGCGCCACCTAAGCAGCGGGCCGGCCC
mRNA            TCCATCGCCATCGTCATCGTCATCGCCAGCGCCAGCGCCACCTAAGCAGCGGGCCGGCCC
                ************************************************************

RC              CAGAGCGGACGAGTCTCTCGAGACCTTTGAAGATAGAACATTACGCGCATTATTCAGCAT
mRNA            CAGAGCGGACGAGTCTCTCGAGACCTTTGAAGATAGAACATTACGCGCATTATTCAGCAT
                ************************************************************

RC              CACTCTCGACGAGACCCAGCAGAAGAATATACACGGCCAGAAATTAACTTTTCTGCCGGG
mRNA            CACTCTCGACGAGACCCAGCAGAAGAATATACACGGCCAGAAATTAACTTTTCTGCCGGG
                ************************************************************

RC              CGTACTCAGTGAACTCAAGGATGAAGGATCGGAAATTAGGATATCTACTGGCGTCTTGGA
mRNA            CGTACTCAGTGAACTCAAGGATGAAGGATCGGAAATTAGGATATCTACTGGCGTCTTGGA
                ************************************************************

RC              TCAGGCGATTTTGGAAGCGGCGTCAAACACTGGCCGTGATACACCCTTGGGTTATCTTTT
mRNA            TCAGGCGATTTTGGAAGCGGCGTCAAACACTGGCCGTGATACACCCTTGGGTTATCTTTT
                ************************************************************

RC              GTCGTGCTGGAAGCGTGTGCGACGGTTGATTAAAGGCTTTCGGAAGTCGTCTGACGATGA
mRNA            GTCGTGCTGGAAGCGTGTGCGACGGTTGATTAAAGGCTTTCGGAAGTCGTCTGACGATGA
                ************************************************************

RC              CCCCCGTTTCGCCGTGATCAGTGAGGCCAAACGCCTATGTATAAGTTATGCTGTTTTTGC
mRNA            CCCCCGTTTCGCCGTGATCAGTGAGGCCAAACGCCTATGTATAAGTTATGCTGTTTTTGC
                ************************************************************

RC              AGTGACTATGCCGGAGATGTTTGGGTTGGTCTCTCGTTTTTCCTTCTTAGCAATATGGCC
mRNA            AGTGACTATGCCGGAGATGTTTGG-----------------------------------
                ***********************
```

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RC              CGGCTGAAAAGGGTGGTGTGATTATAACTCATTGCTGACAAGAGCTTTTGAATAGTGAAA
mRNA            ------------------------------------------------------TGAAA
                                                                      *****

RC              CGCCGACTGGTCGTTCGCCTTTGATCCCAAACCTCCTTCTCGATGCGGAAGACCCTCACG
mRNA            CGCCGACTGGTCGTTCGCCTTTGATCCCAAACCTCCTTCTCGATGCGGAAGACCCTCACG
                ************************************************************

RC              GAGTGGATCTTGAGTTTCTTGCAGAAGTTGTGAAGCTTTTCGAAGAACAGGATGATCTGA
mRNA            GAGTGGATCTTGAGTTTCTTGCAGAAGTTGTGAAGCTTTTCGAAGAACAGGATGATCTGA
                ************************************************************

RC              AGCCGGCAATTATTACTACGGTGGAGCAGATGAGTCAGGAACTATCTGCAAAGACTATGA
mRNA            AGCCGGCAATTATTACTACGGTGGAGCAGATGAGTCAGGAACTATCTGCAAAGACTATGA
                ************************************************************

RC              ATGATGACTATAAGCCATATGTAGCGGTTCGTATTTTTTTACCCTGGTCTCACGATTCTT
mRNA            ATGATGACTATAAGCCATATGTAGCGG--------------------------------
                ***************************

RC              GTACTAAGCGTTTGGTAGGCACTCAGGAACCTCGTTCACCATGCCGCTATCGGATCTGCC
mRNA            ------------------CACTCAGGAACCTCGTTCACCATGCCGCTATCGGATCTGCC
                                   ****************************************

RC              ATTGCTGAGTCTCCGCGATTCCTCAACCAAACAGATGCTGCGTCTTTTGAAGTTAATACA
mRNA            ATTGCTGAGTCTCCGCGATTCCTCAACCAAACAGATGCTGCGTCTTTTGAAGTTAATACA
                ************************************************************

RC              TTACTTGGACCTTGGTTTCGTTTGTCACCGCTTCAAGCTCCAGTCACGACTACTTATTTT
mRNA            TTACTTGGACCTTGGTTTCGTTTGTCACCGCTTCAAGCTCCAGTCACGACTACTTATTTT
                ************************************************************

RC              TCCAGCCCCAAGACCCGGGATCAGGGTTTTATTCTTAACTCGCAGCGGTCCCTTCGAATG
mRNA            TCCAGCCCCAAGACCCGGGATCAGGGTTTTATTCTTAACTCGCAGCGGTCCCTTCGAATG
                ************************************************************

RC              ACACAGCAATTGCTCAGTTCTGATCTTTTGGATGTTATTAATCATCTTATTCGCGCTTCG
mRNA            ACACAGCAATTGCTCAGTTCTGATCTTTTGGATGTTATTAATCATCTTATTCGCGCTTCG
                ************************************************************

RC              AAAGAGGCTAGAGAGAAAGTGCTCGATTGGTTTGCGGCAGCCATTAATCTGAACCACAAG
mRNA            AAAGAGGCTAGAGAGAAAGTGCTCGATTGGTTTGCGGCAGCCATTAATCTGAACCACAAG
                ************************************************************

RC              AGGAGAGCGATGCAGGTTGATCCGAAAACCGTTTCGTCCGATGGGTTCATGTTCAATATC
mRNA            AGGAGAGCGATGCAGGTTGATCCGAAAACCGTTTCGTCCGATGGGTTCATGTTCAATATC
                ************************************************************

RC              ACTACTTGTCTCGACCAGCTCTGTGAGCCGTTTATGGATGCAGCATTCACAAAGGTATGC
mRNA            ACTACTTGTCTCGACCAGCTCTGTGAGCCGTTTATGGATGCAGCATTCACAAAG------
                ****************************************************

RC              TATTTTATCCCGAATTACTCCGTATCATACTAATTCAAGCAGATCGACCGCATAGATGCG
mRNA            ------------------------------------------ATCGACCGCATAGATGCG
                                                           ******************

RC              GGTTATCTTCACCGGAATCCCCGTGTCAAAATGGGCGACGAGACCAAGATAAACGCAGAC
mRNA            GGTTATCTTCACCGGAATCCCCGTGTCAAAATGGGCGACGAGACCAAGATAAACGCAGAC
                ************************************************************

RC              CAACATACATCCGACGAGTTTTATGCACGCAAAGTAGAGGGCACCTCCAACTTTATATCT
mRNA            CAACATACATCCGACGAGTTTTATGCACGCAAAGTAGAGGGCACCTCCAACTTTATATCT
                ************************************************************
```

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RC              GAAGTTTTCTTTCTTACTGTCGCAGCGCATCATTATGGCAGCGAATCTCTGACTTCCAAG
mRNA            GAAGTTTTCTTTCTTACTGTCGCAGCGCATCATTATGGCAGCGAATCTCTGACTTCCAAG
                ************************************************************

RC              CTGGAGCAACTTGAACAGGACCTTCGTCATATGGAAACTCAAATCAACAAGTTTGAGCTT
mRNA            CTGGAGCAACTTGAACAGGACCTTCGTCATATGGAAACTCAAATCAACAAGTTTGAGCTT
                ************************************************************

RC              GAGCGACATAAATGGATCCACAACCCGATGCAATTGAGAACATTTGAAGAAGCCCTCAAA
mRNA            GAGCGACATAAATGGATCCACAACCCGATGCAATTGAGAACATTTGAAGAAGCCCTCAAA
                ************************************************************

RC              AAGTACAAGGACCGACTTGACCTTGGCCTTTGTCTCAAATACAGCCTTCAAGGTTTACTA
mRNA            AAGTACAAGGACCGACTTGACCTTGGCCTTTGTCTCAAATACAGCCTTCAAGGTTTACTA
                ************************************************************

RC              TTTGACACAGTGTGGCAGACTCGTTCCATGCAGTTCATGAGATATGTCATTGTATGGATT
mRNA            TTTGACACAGTGTGGCAGACTCGTTCCATGCAGTTCATGAGATATGTCATTGTATGGATT
                ************************************************************

RC              CTCCGCCTCGTTTCTGGACTGGACTTTCCCAAACAGAAGCTCTCCTTGCCCCTACCGGAA
mRNA            CTCCGCCTCGTTTCTGGACTGGACTTTCCCAAACAGAAGCTCTCCTTGCCCCTACCGGAA
                ************************************************************

RC              GAGCCTCGCGAAATTTTCAAGTGTCTCCCTGAATATTTTATTGATGACATCGTCAGCAAC
mRNA            GAGCCTCGCGAAATTTTCAAGTGTCTCCCTGAATATTTTATTGATGACATCGTCAGCAAC
                ************************************************************

RC              TTCAAGTTCATCATGTGGTCTATGCCACAGATCATCACCACGGCGCAAGGTGATGAACTT
mRNA            TTCAAGTTCATCATGTGGTCTATGCCACAGATCATCACCACGGCGCAAGGTGATGAACTT
                ************************************************************

RC              GTAATGCTGTGCATTGCATTCCTTGAAAGCTCGCAATACATCAAAAACCCCTATCTCAAA
mRNA            GTAATGCTGTGCATTGCATTCCTTGAAAGCTCGCAATACATCAAAAACCCCTATCTCAAA
                ************************************************************

RC              GCCGGTCTTATCTCTATACTGTTCCGAGGTACTTGGCCTCGACCCGGCGGGGCTAGAGGC
mRNA            GCCGGTCTTATCTCTATACTGTTCCGAGGTACTTGGCCTCGACCCGGCGGGGCTAGAGGC
                ************************************************************

RC              ATTCTTGTGGATTTGCTCAATTCATTGCCCTTCGCAAATGAGTATCTCCTTCACTCCGCC
mRNA            ATTCTTGTGGATTTGCTCAATTCATTGCCCTTCGCAAATGAGTATCTCCTTCACTCCGCC
                ************************************************************

RC              ATGAAATTCTACATTGAAGTTGAGCACACTGGAACACATACACAGTTTTTCGACAAGTTC
mRNA            ATGAAATTCTACATTGAAGTTGAGCACACTGGAACACATACACAGTTTTTCGACAAGTTC
                ************************************************************

RC              AATATTCGATACGAAATCTTCCAGATCATCAAGTGCATCTGGAGCAACCCAACTTATAGA
mRNA            AATATTCGATACGAAATCTTCCAGATCATCAAGTGCATCTGGAGCAACCCAACTTATAGA
                ************************************************************

RC              AATCAACTTTCCGATCAAGCAAATGAGAATCTCGACTTCTTCGTTCGATTTGTCAACCTT
mRNA            AATCAACTTTCCGATCAAGCAAATGAGAATCTCGACTTCTTCGTTCGATTTGTCAACCTT
                ************************************************************

RC              CTCTTAAACGACGTGACATTCGTTCTTGATGAAGCATTTACTGCATTTATTACCATCCAC
mRNA            CTCTTAAACGACGTGACATTCGTTCTTGATGAAGCATTTACTGCATTTATTACCATCCAC
                ************************************************************
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RC              GATACGCAAGAGCTACTCAACCGAGAAGGAAACACCATGGAGCAGACAGTACGGCAAGAA
mRNA            GATACGCAAGAGCTACTCAACCGAGAAGGAAACACCATGGAGCAGACAGTACGGCAAGAA
                ************************************************************

RC              AAGGAAGAGGCCCTGAGTGCAGCTCAACGCCAAGCCAAATCCTACATGCAACTCACCAAC
mRNA            AAGGAAGAGGCCCTGAGTGCAGCTCAACGCCAAGCCAAATCCTACATGCAACTCACCAAC
                ************************************************************

RC              GAAACAGTAGCCATGCTCAAACTCTTCACAGAGGCATTAGCAGATTCATTCACTATGCCC
mRNA            GAAACAGTAGCCATGCTCAAACTCTTCACAGAGGCATTAGCAGATTCATTCACTATGCCC
                ************************************************************

RC              GAAATCGTACAGCGATTGGCAGACATGTTGGACTACAACCTTGAGGCGATGGTGGGACCT
mRNA            GAAATCGTACAGCGATTGGCAGACATGTTGGACTACAACCTTGAGGCGATGGTGGGACCT
                ************************************************************

RC              AAGAGCTCGAACCTGCATGTGGGTAATCTGCAAGAATACGGCTTCAATCCCCGTGGACTT
mRNA            AAGAGCTCGAACCTGCATGTGGGTAATCTGCAAGAATACGGCTTCAATCCCCGTGGACTT
                ************************************************************

RC              TTGAGTGAGATCATCGATGTGTATATCAACTTGATGAACAAGGAGAACTTCATCTATGCC
mRNA            TTGAGTGAGATCATCGATGTGTATATCAACTTGATGAACAAGGAGAACTTCATCTATGCC
                ************************************************************

RC              GTGGCCCGGGATGGACGTTCGTACAAACCACAGAATTTTGAGAAGGCAGCTGAAATCATC
mRNA            GTGGCCCGGGATGGACGTTCGTACAAACCACAGAATTTTGAGAAGGCAGCTGAAATCATC
                ************************************************************

RC              CGCAAACGAGCGCTGAAATCAGATGAAGAATTGGCTAAATTAGTGGAATTAGCGAAACGG
mRNA            CGCAAACGAGCGCTGAAATCAGATGAAGAATTGGCTAAATTAGTGGAATTAGCGAAACGG
                ************************************************************

RC              ATCAAGAATGCCAAGGAGGCAGATGATCAGGCAGAGGAGGATTTGGGAGAAATTCCAGAT
mRNA            ATCAAGAATGCCAAGGAGGCAGATGATCAGGCAGAGGAGGATTTGGGAGAAATTCCAGAT
                ************************************************************

RC              GATTTCCTTGGTATGGTTCTCTCTTTCGAGACTCGAAAAACATTTTACTAACATATAGAA
mRNA            GATTTCCTTG----------------------------------------------------
                **********

RC              GACCCCCTGATGTACACCCTCATGGAAGACCCAGTCATCCTACCCAACTCCAAAGTCACG
mRNA            -ACCCCCTGATGTACACCCTCATGGAAGACCCAGTCATCCTACCCAACTCCAAAGTCACG
                 ************************************************************

RC              ATTGACCGCGCCACAATCCGTTCTCACTTGCTGAGCGATCCACATGATCCTTTTAACCGA
mRNA            ATTGACCGCGCCACAATCCGTTCTCACTTGCTGAGCGATCCACATGATCCTTTTAACCGA
                ************************************************************

RC              GCGCCATTGAAAATTGAAGAAGTCGTACCCAGTAAGCACCTAATAATCCTCAAAAACGCA
mRNA            GCGCCATTGAAAATTGAAGAAGTCGTACCCA------------------------------
                ********************************

RC              AAAAGTAGCATTACTGACTGATTTGCGATAGATATGGACCTAAAGAAACAAATTGAAGAC
mRNA            --------------------------------ATATGGACCTAAAGAAACAAATTGAAGAC
                                                ****************************

RC              TTCAAAGCAGAGCGCAAAGCTGCGAAATTGCAGTCCATGAAGAAGGATGTTATGGATACT
mRNA            TTCAAAGCAGAGCGCAAAGCTGCGAAATTGCAGTCCATGAAGAAGGATGTTATGGATACT
                ************************************************************

RC              TCGACTGGGTGATTGCATAGGAAAGATATGGGTCACGGTAGCATTATCATCATTATAACT
mRNA            TCGACTGGGTGA--------------------------------------------------
                ************
```

Appendix E: Primer pair sequences

Primer	Sequence (5' to 3')	Study
LBa1	TGGTTCACGTAGTGGGCCATCG	Inverse PCR
RB1	TAGCTGATAGTGACCTTAGGCGA	Inverse PCR
LBb1	TGGTTCACGTAGTGGGCCATCG	Sequencing
RB2	TCTGACGTATGTGCTTAGCTCA	Sequencing
Gpda-F	CAAGGTCGTTGCGTCAGTC	Southern Blot
Cycl-R	GCGTGAATGTAAGCGTGAC	Southern Blot
ChsB-F	GCGTTGCAATTTATCCTCTCG	DNA contam.
ChsB-R	TCCACCGCCTTTCAGTTTATC	DNA contam.
Ufdb5 RT-F	TAACTTTTCTGCCGGGCGTA	RT-PCR
Ufdb5 RT-R	GATCAAAGGCGAACGACCAG	RT-PCR
Ufdb3 RT-F	CCCCCTGATGTACACCCTCA	RT-PCR
Ufdb3 RT-R	AGCTTTGCGCTCTGCTTTGA	RT-PCR
Btub-F	GCTCCGGTGTCTACAATGGC	RT-PCR
Btub-R	AGTTGTTACCAGCACCGGAC	RT-PCR

Appendix F: Minimal Media recipe

For 100 ml:

NH_4Cl	0.2 grams
$(NH_4)_2SO_4$	0.1 grams
KCl	0.05 grams
NaCl	0.05 grams
KH_2PO_4	0.1 grams
$MgSO_4 \cdot 7H2O$	0.05 grams
$FeSO_4 \cdot 7H2O$	0.002 grams
Agar	1.5 grams
$C_6H_{12}O_6$	0.05 grams (0.05%)
	1 gram (1%)
	2 grams (2%)

Appendix G: Two-way repeated measures ANOVA output for radial growth on MEA, PDA, and SDA

Tests of Within-Subjects Effects

Measure:MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Day	Sphericity Assumed	85.607	5	17.121	11531.757	.000
	Greenhouse-Geisser	85.607	3.732	22.939	11531.757	.000
	Huynh-Feldt	85.607	5.000	17.121	11531.757	.000
	Lower-bound	85.607	1.000	85.607	11531.757	.000
Day * Medium	Sphericity Assumed	1.991	10	.199	134.097	.000
	Greenhouse-Geisser	1.991	7.464	.267	134.097	.000
	Huynh-Feldt	1.991	10.000	.199	134.097	.000
	Lower-bound	1.991	2.000	.995	134.097	.000
Day * Strain	Sphericity Assumed	.288	5	.058	38.810	.000
	Greenhouse-Geisser	.288	3.732	.077	38.810	.000
	Huynh-Feldt	.288	5.000	.058	38.810	.000
	Lower-bound	.288	1.000	.288	38.810	.000

Day * Medium * Strain	Sphericity Assumed	.174	10	.017	11.706	.000
	Greenhouse-Geisser	.174	7.464	.023	11.706	.000
	Huynh-Feldt	.174	10.000	.017	11.706	.000
	Lower-bound	.174	2.000	.087	11.706	.000
Error(Day)	Sphericity Assumed	.178	120	.001		
	Greenhouse-Geisser	.178	89.567	.002		
	Huynh-Feldt	.178	120.000	.001		
	Lower-bound	.178	24.000	.007		

Tests of Between-Subjects Effects

Measure:MEASURE_1

Transformed Variable:Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	610.144	1	610.144	79656.181	.000
Medium	2.995	2	1.497	195.472	.000
Strain	.567	1	.567	73.987	.000
Medium * Strain	.467	2	.234	30.508	.000
Error	.184	24	.008		

Appendix H: Two-way repeated measures ANOVA output for radial growth on MM supplemented with 0.05%, 1%, and 2% glucose

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
factor1	Sphericity Assumed	81.012	7	11.573	3244.993	.000
	Greenhouse-Geisser	81.012	2.194	36.920	3244.993	.000
	Huynh-Feldt	81.012	3.824	21.186	3244.993	.000
	Lower-bound	81.012	1.000	81.012	3244.993	.000
factor1 * Glucose2	Sphericity Assumed	.039	14	.003	.781	.687
	Greenhouse-Geisser	.039	4.388	.009	.781	.558
	Huynh-Feldt	.039	7.648	.005	.781	.616
	Lower-bound	.039	2.000	.019	.781	.480
factor1 * Strain2	Sphericity Assumed	2.661	7	.380	106.570	.000
	Greenhouse-Geisser	2.661	2.194	1.213	106.570	.000
	Huynh-Feldt	2.661	3.824	.696	106.570	.000
	Lower-bound	2.661	1.000	2.661	106.570	.000
factor1 * Glucose2 * Strain2	Sphericity Assumed	.014	14	.001	.275	.995
	Greenhouse-Geisser	.014	4.388	.003	.275	.905
	Huynh-Feldt	.014	7.648	.002	.275	.968
	Lower-bound	.014	2.000	.007	.275	.764
Error(factor1)	Sphericity Assumed	.300	84	.004		
	Greenhouse-Geisser	.300	26.331	.011		
	Huynh-Feldt	.300	45.887	.007		
	Lower-bound	.300	12.000	.025		

****factor1 = Day

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	252.545	1	252.545	6558.429	.000
Glucose2	.000	2	.000	.006	.994
Strain2	6.418	1	6.418	166.665	.000
Glucose2 * Strain2	.015	2	.007	.190	.830
Error	.462	12	.039		

Appendix I: Permissions

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Lan-Ying Lee and Stanton B. Gelvin*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907–1392

Plant Physiol. Vol. 146, 2008

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The Journal of Microbiology, August 2006, p.383-395

Vol. 44, No. 4

A Comparison of the Phenotypic and Genetic Stability of Recombinant *Trichoderma* spp. Generated by Protoplast- and *Agrobacterium*-Mediated Transformation

Rosa Elena Cardoza[1,2], Juan Antonio Vizcaino[1], Maria Rosa Hermosa[1], Enrique Monte[1] and Santiago Gutie´rrez[2,*]
[1]Spanish-Portuguese Center of Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, 37007 Salamanca, Spain
[2]University of León, Campus of Ponferrada, Superior and Technical University College of Agricultural Engineers, Area of Microbiology, Avda. Astorga s/n. 24400 Ponferrada, Spain.
(Received December 24, 2005 / Accepted July 7, 2006)

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