Chitin Synthase Gene Expression in the Dimorphic Fungus *Penicillium marneffei*

by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

April, 2011

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ABSTRACT

Penicillium marneffei is a pathogenic fungus endemic to Southeast Asia. It is the only *Penicillium* species that displays thermally dependent dimorphism. At 25° C, the fungus grows as a filamentous mold, but develops as a fission yeast at 37° C. The pathogenicity of *P. marneffei* is associated with this dimorphism. Presumably, dimorphism directly reflects changes in the cell wall structure.

A major fungal cell wall component is chitin. Seven chitin synthase genes have been identified in *P. marneffei* and each is presumed to serve different functions during cell wall development, hyphal growth, conidiophore development, and septum formation. Determining the temporal and spatial expression of these *chs* genes would provide a better understanding into the growth and morphogenesis of *P. marneffei*. Hence, using a reverse-transcription polymerase chain reaction and a reverse-transcription quantitative polymerase chain reaction, the expression of each *chs* orthologue was assessed after 1 and 5 days of incubation of the fungus at 25° C and 37° C under conditions that do not induce conidiogenesis. The results indicate that some *chs* genes are differentially expressed, some appear constitutively expressed at various levels, and some are not expressed under the experimental conditions employed. These patterns of expression appear to correlate with the purported function of each gene and the developmental phase examined.

ACKNOWLEDGEMENTS

Dr. Chester R. Cooper Jr. – I would like to express my gratitude to you for the honor of allowing me to work in your laboratory. You have taught me invaluable technical skills and as well as work ethic ("Daylight is burning!"). I can't thank you enough for your guidance, encouragement and confidence in me. You believed in me when I doubted myself.

Dr. Asch, Dr. Walker and Dr. Caguiat – Thank you for answering my endless questions and for opening your labs to me. I could not have accomplished what I did in my research without your guidance as committee members.

Julie Chandler –Thank you for providing me with some amazing bench skills and answering all my questions even when you were no longer at YSU. Without your patience, encouragement and guidance none of this would be possible.

Dr. Lorna Gallagher – Thank you for all your guidance and invaluable input. Special thanks for reviewing my thesis and providing me with great feedback.

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Introduction

Background

Penicillium marneffei is a dimorphic fungus endemic to Southeast Asia. (Cao, 2007; Cooper, 2008). It was first discovered in 1956 by Capponi and Sureau while using bamboo rats as a model for rickettsiosis. Biopsies taken from dead bamboo rats grew a fungus with *Penicillium*–like features, whereas *in vivo* observations only detected a yeast form (Cooper, 2008). In 1959, Segretain studied the isolate and designated it as a new species, *Penicillium marneffei.* However, no human cases of infection due to *P. marneffei* (penicilliosis) were documented until 1973.

With the AIDS epidemic emerging in the late 1970's, human cases of penicilliosis markedly increased (Cooper, 2008). As of 2008, more than 6000 cases were documented in northern Thailand alone involving a high percentage of HIV- infected individuals and those with compromised immune systems (Cooper, 2008; Wu, 2008). The rate of co-infection with HIV is so high that *P. marneffei* is now considered an AIDS defining pathogen (Cooper, 2008; Wu, 2008).

Unfortunately the origin of *P.marneffei* remains unknown despite many environmental studies. It is believed that the bamboo rat plays an important role in the life cycle of *P. marneffei* and creates a widespread aerosol-dispersal when the animal expires. A current study investigated the possibility that the bamboo rat is the ultimate source of penicilliosis infections in humans. Investigators examined similarities of the genotypes of *P. marneffei* isolates obtained from humans and bamboo rats living in different regions. The study sought to determine if humans and bamboo rats encountered

the same unknown reservoir or if the rats' act as a vector for human infection (Cao *et al*, 2011). Despite determining molecular similarities in human and rat *P. marneffei* isolates, the study was unable to ascertain if the rats were the ultimate source of human infection. The origin of *P. marneffei* remains enigmatic.

Continued research is needed to determine real and potential amplifiers of human infections by *P. marneffei* within the environment. However, extensive documentation has suggested the infection route of *P. marneffei*. In most fungal pathogens, such as *P. marneffei*, infection begins with inhalation of spores, termed conidia, produced by the filamentous mold form. Once in the host, the conidia are phagocytized by pulmonary histiocytes and grow as yeast cells (Cooper, 2008). Conceivably, the phagocyte protects the yeast cells and aids in the dissemination of the fungus throughout the host.

Penicillium marneffei infections affect the host's organs and soft tissues. Infections in patients with full-blown AIDS show symptoms of weight loss, skin lesions, anaemia, and hepatomegaly (Wu, 2008). If left untreated, *P. marneffei* infections are fatal. Initial treatments in the 1980s and 1990s began with doses of fluconazole and amphotericin B (Wu, 2008). It appears that *P. marneffei* is resistant to fluconazole, although amphotericin B seems to have some moderate affect on infections. Currently, amphotericin B is administered first to infected individuals followed by prolonged prophylactic doses of itraconazole or voriconazole (Cooper, 2008; Wu, 2008).

Morphology

Penicillium marneffei's dimorphic ability appears to be an essential factor contributing to the pathogenicity of the organism. It is the only species of *Penicillium*

that exhibits thermally dependent dimorphism, i.e., it alternates between a filamentous form at 25° C and yeast form at 37° C (Andrianopoulos, 2002; Cao, 2007). Based on temperature cues, dimorphism can occur in both hyphal-yeast and yeast-hyphal directions (Andrianopoulos, 2002).

In the filamentous stage, *P. marneffei* displays asexual development via the production of conidia on complex multicellular structures termed conidiophores. This filamentous stage begins with the development of multinucleate aerial stalk cells produced by hyphal cells. These stalk cells may be septate and produce rama, i.e., secondary stalks (Andrianopoulos, 2002). The stalk cell tips then produce metulae and phialides. Metulae are first to bud from the stalk cells which, in turn, form phialides that subsequently produce conidia. The conidia form a chain as new growth displaces the older spores in a basipetal mode.

Direct morphogenesis to yeast cells does not occur when the conidia are germinated at 37° C. Germination of conidia in both the mold and yeast forms initiates with isotropic swelling. Subsequently, both forms polarize to form a germ tube, which then elongates to form hyphae. The only distinguishable characteristic at this time is that the hyphae at 37° C are broader and highly branched. At 18-24 hours, the hyphae generated at 37° C are shorter and wider than the hyphae produced at 25° C. The former also possess double septa. After 72 hours of incubation at 37° C, the yeast cells morphology begins to form. These yeast cells are unique as they divide by fission compared to the budding of other fungal dimorphic pathogens (Andrianopoulos, 2002; Cooper 2008). The cell wall matter between the double septa degrades to liberate the uninucleated single cells termed arthroconidia.

Chitin

The cell walls of filamentous fungi are comprised of mainly chitin and glucan, both of which are critical for morphogenesis (Ruiz-Herrera, 2002). The cell wall rigidity, integrity and shape of fungi is partly due to chitin, which is the product of the enzyme chitin synthase (Ruiz-Herrera, 2002). Chitin is important to the cell wall structure and is greatly dispersed throughout the fungal kingdom. (Roncero, 2002)

Chitin synthase activity is localized to the cell plasma membrane (Mellado, 1995). Chitin is a β-1, 4 linked homopolymer of *N*-acetylglucosamine (GlcNAc) that is formed by hydrogen-bonded, anti-parallel chains termed microfibrils, which are extremely strong and crystalline. Weight for weight, chitin is said to be the strongest biological polymer and stronger than steel (Munro & Gow, 2001). Chitin synthesis, degradation, assembly, and cross-linking to other cell wall components are of great importance in many filamentous fungi (Horiuchi, 2009; Mellado, 2003).

The deposition and formation of chitin is a complex multi-faceted, inter-connected biophysical and biochemical series of events which begin within the cell and finishes with chitin inclusion in the cell wall (Cohen, 2001). The process begins with the biotransformations of sugars which encompass phosphorylation and amination, then formation of UDP-N-acetylglucosamine substrate. This is followed by the synthesis of chitin synthase units termed chitosomes (Cohen, 2001).

Chitosomes are cytoplasmic microvesicles which originate from the endoplasmic reticulum. They have been observed, through electron microscopy, to be in abundance at the hyphal tip. This observation suggests the involvement of chitosomes in the

trafficking of chitin synthase. Eventually these chitosomes fuse with the plasma membrane prior to their proteolytic activation. Some theories of chitin translocation across the cell membrane are explained by a mechanism involving polymerization within the chitosome before they fuse with the cell membrane.

Chitin synthases (CHS) are membrane-bound proteins which can be categorized through genetic studies into classes based on amino acid sequence similarities (Horiuchi, 2009; Mellado, 2003). Different fungi contain various numbers of chitin synthase encoding genes (*chs*) per genome, i.e., *Saccharomyces cerevisiae* contains three members, whereas *Benjaminiella poitrasii* has as many as eight.

The classes of chitin synthases are divided into three divisions. Division I contains CHS classes I, II, and III. A hydrophobic C-terminus and a conserved catalytic subdomain (pfam01644), which is surrounded by a hydrophyllic N-terminus region, are characteristic of Division I (Riquelme, 2008; Bowen *et al,* 1992). Researchers were able to identify transmembrane topology domains of various fungal CHS and determined class I CHS possessed a short C-terminal domain in the cytoplasm and a long N-terminal proposed to be located in the extracellular space (Riquelme, 2008).

Division II consists of classes IV, V, and VII. This division is characterized by a catalytic domain preceded by pfam00173, a cytocrome b5-like binding domain. Division II have a short C-teminus and a long N-terminus domain proposed to be located in the extracellular space (Riquelme, 2008). In their amino terminus, classes V and VII also have a myosin motor domain. Myosins are motor proteins that travel along actin filaments (Horiuchi, 2009).

Division III was created for class VI because it lacked the characteristics of Division I or II. CHS class VI was observed to posses a long C-terminal and a very short N-terminal both proposed to be located in the extracellular space (Riquelme, 2008).

Research has provided some proposed functions of chitin synthases from various organisms including *Saccharomyces cerevisiae, Canidia albicans, Aspergillus fumigatus,* and *Wangiellia dermatitidis*. Class I genes in these organisms have been proposed to provide repair functions in concert with cytokinesis. Experimental procedures have yielded possible functions of Class II *chs* including synthesis of chitin in the primary septum, maintenance of lateral wall and the septum integrity and conidiogenesis. Class III *chs* genes were mainly proposed to have a role in hyphal growth. Class IV and Class V *chs* are proposed to have roles in bulk chitin synthesis and hyphal tip growth. Class IV is also proposed to contribute to the cell wall rigidity of germinating conidia, as well as the subapical region of hyphae and conidiophores (Borgia, 1996; Horiuchi, 2009; Spect, 1996). Class VI *chs* genes are proposed to perform compensatory functions with Class V genes which are essential for hyphal tip growth. Class VII has purported functions in conidiation (Borgia, 1996; Horiuchi, 2009; Spect, 1996).

Gene Deletion Studies

The *chs* genes in *Penicillium marneffei* have not been previously studied. However, in similar species, such as *Aspergillus nidulans* and *Aspergillus fumigatus,* the *chs* have been the subject of extensive research. The existence of multiple *chs* points to the possibility that each possesses a different function and cellular location during specific points in the cell cycle. However, the existence of multiple genes in the same

location poses the possibility of a need to form multiunit complexes or a redundancy of function (Riquelme, 2008). Single deletions of known *chs* have been performed in *Aspergillus nidulans* and *Aspergillus fumigatus* to help further understand their functions (Munro, 2003).

In the *A. nidulans* genome, there are eight *chs* genes expressed: *chsA, chsB, chsC, chsD, chsF* (AN4367.3)*, chsG* (AN1046.3)*, csmA* and *csmB* (Horiuchi,2009). *A. fumigatus* possess seven *chs*: *chsA, chsB, chsC, chsD, chsE, chsF, and chsG* (Mellado, 2003). Both species possess class III chitin synthase encoding genes.

Experiments with the deletion mutants of *chs* in *A. nidulans* and *A. fumigatus* have been performed (Horiuchi, 2003). Deletion strains have been the primary means to determine the individual roles of various *chs* (Lenardon, 2010). The effects these deletions had on phenotype, hyphal and conidiophore development, cell wall and septa indicate that certain *chs* have roles in maintaining the cell wall integrity and normal growth (Mellado, 2003). In general, disruptions in classes II, V and VII *chs* showed vast changes in phenotype. Class III deletions displayed no effect to levels of chitin, whereas chitin levels in class V mutants were reduced. Class VI deletion mutant did not produce obvious morphological defects, yet their cell wall chitin level was reduced (Riquelme, 2008). Researchers were able to propose gene functions based on the results of the deletion strains.

Table 1. Chitin synthase gene orthologues in *Aspergillus fumigatus, Aspergillus nidulans* and *P. marneffei* (Borgia, 1996; Horuchi, 2009; Spect, 1996)

		Fungal Species			
Division	**Class**	***Aspergillus nidulans***	***Aspergillus fumigatus***	***Penicillium marneffei*** (GenBank Accession Number)	**Proposed Function**
1	II	*chsA*	*chsB*	*chsB* (XM_002148034)	Required for conidial development
1	III	*chsB*	*chsG*	*chsG* (XM_002153543)	Required for hyphal growth
1	I	*chsC*	*chsA*	*chsA* (XM_002148484; XM_002148485)	Required for hyphal growth and conidiogenesis
1	III	*chsF*	*chsC*	---	
2	IV	*chsD*	*chsF*	*chsF* (XM_002149542)	Contributes to cell wall rigidity of germinating conidia, the subapical region of hyphae, and conidiophores
2	V	*csmA*	*chsE*	*csmA (chsE)* (XM_002145210)	Perform compensatory functions that are essential for hyphal tip growth
2	VI	*csmB*	Afu2g13430	*csmB* (XM_002145211)	
3	VII	*chsG*	*chsD*	*chsD* (XM_002146248)	Required for conidiation

As noted in Table 1, *chsA* and *chsC* in *Aspergillus nidulans* are proposed to function in hyphal growth and are required for conidial development. Subsequently, one might propose that the orthologues *chsB* and *chsA* in *P. marneffei* would display the proposed functions given their sequence similarity (data not given). Experimental results for *Aspergillus nidulans* concluded that the deletion of *chsC* showed no phenotypic change and the deletion of *chsA* caused reduced conidiation. When these two genes were

deleted together, hyphal density was lowered and sensitivity to SDS, KCL, NaCl, sorbitol, chitin binding dyes and chitin synthase inhibitors was increased (Horiuchi, 2009). It can, therefore, be assumed that *chsA* and *chsC* have overlapping roles in the growth of hyphae and conidiation. Observation through transmission electron microscopy (TEM) determined that this mutant strain had thickened cell walls and septa and that the septal pores were larger than the corresponding wild type. TEM also revealed abnormal conidoiophore structure, abnormally extended metulae and undifferentiated phialides in experiments where *chsA* and *chsC* were deleted together.

A. nidulans chsB and *P. marneffei chsG* are proposed to be required for hyphal growth. Deletions of *chsB* yielded minuscule colonies that displayed highly branched hyphae which could not be corrected with additional osmotic stabilizers added to the growth media. These results suggested that *chsB* is critical in hyphal tip growth (Horiuchi, 2009).

Mellado *et al.* (2003) determined that the deletion of *chsE* and *chsG* in *A. fumigatus* led to an altered phenotype. The *chsG* mutant strain showed a reduction in the rate of radial growth and chitin synthase activity. This strain produced highly branched hyphae, which suggested this enzyme functions in apical tip (Mellado, 2003). The mycelia chitin and conidiation in *A. fumigatus chsE* mutant strains were reduced and exhibited hyphae swellings along the length of the mycelia (Mellado, 2003). Therefore, the suggested functions for *chsE*, *chsG* and the *P. marneffei* orthologues, *csmA* and *chsB*, are they are essential for hyphal tip growth.

It should be noted that *csmB* in both species performs overlapping functions with *csmA* for hyphal tip growth. *CsmA* and *csmB* contain chitin synthase domains at their C-termini and myosin motor like domains at their N-termini (Horiuchi, 2009).

Research on *A. nidulans* and *A. fumigatus* denotes the importance of chitin synthase-encoding genes in the cell wall and suggests similar roles for the orthologues found in *P. marneffei.* Because *P. marneffei* is dimorphic, the expression and role of chitin synthase-encoding genes should also be investigated in the yeast phase. For comparison, one can look to the *chs* orthologues of *Saccharomyces cerevisiae*.

Generally, the dimorphic transition from yeast to hyphae causes changes in the cell wall composition. Therefore, it is not unusual for the chitin content of hyphal cells to be higher than in yeast cells (Munro, 2003). The differentiation patterns in fungi are more complex than that of yeast, so it is reasonable to presume that dimorphic fungi possess more chitin synthase genes (Borgia, 1996; Rancero, 2002). Presumably the *chs* diversity found in fungi could be from the demands in location and regulation of the synthesis of chitin throughout different vegetative and sexual cell cycle stages (Riquelme, 2008). Most of the chitin content in yeast is found in the septum which separates the mother and daughter cells of budding yeast. An example of this difference is the 20% content of chitin in the cell walls of *Aspergillus* versus the 1-2% chitin content found in the *S. cervisiae* cell wall.

Deletion experiments in *S. cervisiae* were performed similar to those performed in *A. nidulans* and *A. fumigatus*. It was determined that this species produces three chitin synthases; *Chs1*, *Chs2* and *Chs3*. The *Chs1* was noted to not be essential for survival of the cell. Chitin synthesized in the septum by *Chs2* and *Chs3* provides the synthesis of

chitin in the cell wall and various other regions other than the septum (Baymiller, 1993). Disruption experiments of *Chs2* verify that it is essential for growth and is more detrimental to the cell viability when deleted then the other two genes (Silverman, 1988; Baymiller, 1993).

qPCR/RT qPCR

Deletion experiments assess the purported functions of the chitin synthase genes, whereas quantitative real time PCR (qPCR) provides quantification of the expression of these genes. qPCR was developed due to the need to quantify differences in mRNA expression and due to the small amount of mRNA available in some procedures. qPCR is now the method of choice for analysis of individual gene expression. Traditionally, PCR/RT-PCR has been used to determine the quantification of gene expression; however, they possess some limitations. The DNA product (genomic or cDNA derived from RNA) in PCR/RT-PCR is amplified for a number of cycles then visualized through agarose gel electrophoresis and ethidium bromide staining. This is not reliable for absolute quantification for several reasons.

PCR goes through exponential and linear phases where initially the reaction is not limited by enzymatic substrates or activity. However, substrates do eventually become limited or exhausted. When this occurs, the reaction reaches a plateau and in theory, the samples will reach the same total amount of amplified DNA. This can obscure differences in initial DNA/cDNA and is variable. In general, the reaction plateau of different samples will be at different levels for many reasons and in many incidences,

the amount of product is not related to the amount of input DNA. Therefore, quantifying PCR products upon completion of the reaction magnifies any minute differences in reaction efficiencies. Also the densitometry of the gels lacks sensitivity and is somewhat limited in its dynamic range (Sellner and Turbett, 1998; Zhoa *et al*., 1996). Hence, this method is more a qualitative tool and should be used to merely detect the presence or absence of DNA.

qPCR yields a measurement of quantification of PCR products accumulated throughout the reaction in "real-time" for each PCR cycle. These reactions permit measurement of a fluorescent detector molecule which eliminates the need for post-processing steps and decreases experimental error (Heid *et al*., 1996).

As mentioned, PCR reactions contain three main phases; exponential, linear, plateau. In the exponential phase, products are small making this phase hard to detect with real-time fluorescence. In the linear phase, products continue to accumulate but eventually the reaction efficiency decreases as reagents become limited. In the plateau phase, accumulation of product ceases. It is important to note that qPCR is a kinetic approach in which the linear phase is examined for quantification (Van Guilder *et al*. 2008).

DNA synthesis is monitored in this phase in qPCR with SYBR-green. SYBR-green is a dye that binds to double stranded DNA and fluoresces much brighter than ethidium bromide. As more DNA is synthesized, more of the SYBR-green will bind to it and the resulting fluorescence level will increase. The fluorescence increase is measured as the dye binds to the increasing amount of synthesized DNA in the reaction (Zipper *et al*. 2004).

To determine if the DNA in a question is the reason for amplification, qPCR programs offer an option to conduct a melting curve. This will determine the melting point of the product at the end of the amplification reactions. DNA double helix melting temperatures depend on its base composition therefore all melting temperatures of PCR products from a specific primer pair should be identical. If there is more than one melting temperature, as displayed by multiple peaks in the melt curve, this could suggest contamination or primer-dimers or amplification of a similar sequence in its genome. Checking the melting temperatures for all samples is an important quality control step as SYBR-green cannot distinguish between one DNA sample and another.

Analysis of qPCR data can be conducted with absolute levels, where the numbers of copies of specific RNA can be calculated. It also can be analyzed using relative levels stating a sample has twice the amount of mRNA of a specific gene sample. Due to simplicity of the latter, this is the commonly used analysis method.

To calculate the number of copies in an absolute quantification, an RNA standard curve of the gene of interest must be created. Serial dilutions of a known number of copies of pure RNA is diluted then amplified. This curve is used to compare with the unknown signal to devise a starting concentration.

In relative quantification, the most common method used is the $2^{-\Delta\Delta CT}$ method ($2^{-\Delta\Delta CT} = 2^{-(CTTG - C\ TCG)\ treated - (CTTG - CTCG)\ control}$. This method compares a gene of interest and an endogenous control's crossing thresholds to give a relative quantification. The crossing threshold (C_t) is the number of cycles in a reaction to reach an amount of fluorescence. Current guidelines (MIQE, minimum information for publication of quantitative real-time PCR experiments) apply the term quantification cycle, C_q,

replacing the use of the term threshold cycle (Lefever et al., 2009). For this paper, however, the use of C_t will remain in use for the sake of understanding the term when applied in previously derived equations.

Two assumptions are made with the $2^{-\Delta\Delta CT}$ method. The first being that the reaction is at 100% efficiency, meaning that each PCR cycle doubles the amount of product. This assumption gives practice to using low cycle numbers in the exponential phase where products do not degrade and substrates are not limited. This yields to the setting of the C_t at the earliest cycle that is possible. The second assumption being there is a gene in all the samples that is being consistently expressed (Livak, 2001).

The C_t values are collected for each reaction and the difference between the gene of interest and the endogenous gene are calculated giving the ΔC_t. The control-condition ΔC_t is then subtracted from the treated-condition ΔC_t providing the $\Delta\Delta C_t$. - $\Delta\Delta C_t$, the negative value from subtraction, is used in the equation as the exponent of 2. This exponent of 2 represents the "corrected" numbers of cycles to the threshold. Once put in the equation, a value is given which is referred to as the RQ, the relative quantity value.

It is important to note that C_t should not be used directly in statistical calculations because it is mathematically incorrect. They are exponents which can not be used for ANOVAs or t-tests which require normal distribution. Therefore the aforementioned method or an alternative method developed by Michael Pfaffl must be used (Livak, 2001).

Michael Pfaffl and his colleagues developed a method based on the PCR efficiency and the C_t (ratio = $(E_{target})^{\Delta Ct_{target}(control - sample)} / (E_{ref})^{\Delta Ct_{ref}(control - sample)}$). The efficiencies (E) is calculated according to $E = 10^{[-1/slope]}$. Both the $2^{-\Delta\Delta CT}$ and the Pfaffl

methods are acceptable standards to calculate the quantification of a gene of interest in qPCR (Pfaffl, M.W., 2001). The iQ5 Optical System software used in the iCycler (BIO RAD) for RT qPCR used in this study, analyzes data using equations derived from these mathematical equations.

Chitin synthase analysis would benefit greatly from qPCR/RT qPCR to determine the differential amounts of expression per gene. Traditionally, PCR/RT-PCR was used to show expression of a gene of interest. However, these methods, at best, represented only qualitative measurement; if a gene was present or not. Using qPCR and either the $2^{-\Delta\Delta CT}$ or the Pfaffl method to calculate quantification provides great improvement to the insight of gene quantification including chitin expression.

Research Applications

Whether using gene deletion to determine functions of a gene and viability or qPCR to quantify its expression, researchers are able to analyze strengths and weaknesses in the organism of interest. This opens up avenues in research, including drug development and application.

Deletion experiments on both mold and yeast forms in *P. marneffei* have shown detrimental effects to the survival of a fungal species when *chs* was deleted. Therefore, inhibition of the synthesis of chitin has been the focus of antifungal drugs. To date, nikkomycins and polyoxins are the most potent CHS inhibitors against class I enzymes, yet they are not as effective on the other CHS enzymes in other classes (Lenardon, 2010). Screening for other class inhibitors was prompted by the observation of required roles of

CHS1 in *C. albicans*. That study reinforced the probability that CHS enzymes have compensatory functions (Lenardon, 2010).

Drugs that can accommodate for overlapping *chs* functions in fungi and cell wall synthesis inhibitors are also potential studies of interest. Fungi show an ability to produce excess chitin in the cell wall when there is cell wall damage. This function was noted when fungi were treated with echinocandin drugs. The drugs became less potent in the presence of the excess chitin and thereby heightening the survival of the fungi (Lenardon, 2010). Research to accommodate for this compensatory function pointed out the possibility that combination therapies would produce more successful fungicidal regimens with less resistance mechanisms when targeting two major structural fungal polysaccharides (Lenardon, 2010). Antagonists of the signaling pathway, which regulate activity and expression of synthase, combined with cell wall synthase inhibitors may also pose as potential antifungal combination therapies (Lenardon, 2010).

Differences in gene expression due to the introduction of inhibitors have been the focus of research utilizing qPCR techniques. The ability to quantify the effects a substance has on a gene of interest gives insight of how the expression of that gene responds to the applied substance. Researchers can see in actual realms of quantification the effects on gene expression.

An example of the use of qPCR to determine inhibiting external effects was applied in *C. albicans.* Investigators introduced a 12-carbon molecule, 2-dodecanol (decyl methyl carbinol), to determine if it would inhibit hyphae production. They examined the expression of *sir2* mRNA in *C. albicans* at different time points of yeast-hypha transitions with and without the presence of 2-dodecanol (Siew-Ying Lim et al.,

2009). They were able to determine any inhibiting effects of 2-dodecanol by the qPCR quantification of hyphae production.

Researchers can also use qPCR to quantify the effects of elicitors that may increase expression. One such study aimed to investigate the effects of single and multiple additions of oligosaccharide elicitors on the production of penicillin G in *Peniillium chrysogenum.* Investigators were able to determine the enhancement of penicillin G transcript copy number of penicillin biosynthetic genes corresponding to the increased production of penicillin G by analyzing the quantification determined through qPCR (Nair *et al*., 2009).

Data from this research and any further research on normal chitin expression will aid investigators when designing gene deletion and qPCR experiments. qPCR coupled with deletion experiments could enhance the knowledge of the viability of the organism and yield solid data on the effects of gene deletion or an introduced substance. Determination of the time frames where chitin is being expressed and at what levels combined with the proposed function will aid in pinpointing *chs* expression which are crucial for cell survival. It is conceivable then, that investigators can use gene deletion along with qPCR to investigate changes in morphology, cell wall viability and changes in *chs* expression.

AIM OF RESEARCH

Chitin appears to play an imperative role in cell wall development and viability in both yeast and mold phases. Deletion of any *chs* could prove detrimental to the survival of this species. Therefore, it would prove useful to investigate normal expression of

chitin during the developmental phases and correlate this expression with possible gene functions. This would provide a benchmark of chitin gene expression upon which to base further research. Establishment of normal gene expression could then give way to analyzing effects of new novel drugs on the various CHS isozynes in *P. marneffei*. Therefore, this study will aim to investigate normal expression of all seven chitin synthase genes in *P. marneffei* utilizing RT-PCR to verify expression and subsequently RT qPCR to quantify expression.

RESEARCH GOALS

This study seeks to identify the roles and expression of chitin synthase (CHS) during cellular development of *Penicillum marneffei*. The goals are as follows:

1. Determine differential *chs* expression and levels in the mold phase of *P. marneffei*.
2. Determine differential *chs* gene expression and levels in yeast phase of *P.marneffei*.

Little is known of the *chs* expression in *P. marneffei.* Therefore, analysis of the expression could lead to a clearer understanding of the role of *chs* genes in morphogenesis and virulence. This could prove important to understanding the pathogenicity of *P. marneffei* and in developing new antifungal drugs that target the cell wall.

HYPOTHESIS

Based on previous research with similar species, the expression of chitin synthase genes in the mold and yeast forms of *Penicillium marneffei* should show different expression at different time frames. This expression should correlate with the purported function of each gene and the developmental phase examined.

Chapter II

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), Fisher-Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO). The potato dextrose agar (PDA; Difco brand) and Sabouraud dextrose broth (SAB; Difco 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_20).

P.marneffei Growth

Penicillum marneffei F4 was grown 8-10 days on potato dextrose agar (PDA) in 150 cm^2 cell culture flasks with vented lids at 25° C. Strain F4 was obtained as a generous gift from Chiang Mai University, Thailand and maintained at Youngstown State University.

Conidia Harvesting

Conidia and hyphae were collected from PDA plates by pippetting 10 ml of ddH_20 and gently scrapping the plate. The suspension was then be passed through a screen cap (Bio-Rad) separated with 1 inch glass wool (Corning, Acton, MA) which was secured to the end of a sterile 50-ml conical centrifuge tube. The cell suspension was

placed in the top of the screened cap, capped, and then centrifuged for 30 seconds at 150-200 x g, thus isolating the conidia from the hyphae via the glass wool. Final concentrations of conidia were determined using a hemocytometer.

Broth Inoculation

A water suspension containing conidia at 5 x 10^8 was centrifuged, the supernatant discarded and the remaining conidial pellet resuspended in a small amount of SAB pre-warmed to 25° C or 37° C. Six separate 500 ml Erlenmeyer flasks containing 50 ml SAB were inoculated with final concentrations of 1 x 10^7 conidia per ml. The inoculated SAB broth was cultured in a shaking water bath at 25°C and 37° C. Two flasks were collected from each temperature after 24 hours and one flask from each temperature after 120 hours. Cells from these cultures were collected at the respected time frame by washing the mold and yeast cultures with TE buffer prior to centrifugation in the Sorvall at 15,000 rpm at 4° C for fifteen minutes several times. The washing was repeated several times. The pellets were weighed to ensure at least a 200-400 mg cellular material was obtained.

RNA Extraction

Using the protocol from Ameresco, one-tenth of the pelleted cells were broken using a bead-beating technique with glass beads and a mini bead beater machine (Biospec). Approximately 0.8 g of acid washed glass beads (0.5 mm) and 1 ml of Ribosol (Ameresco) was added to the pellet. The tubes were then placed in the bead beater machine five times for 30 seconds at 5,000 rpm and cooled on ice 30 seconds between each operation of the bead beater.

The tubes were then centrifuged at 4° C for two minutes at 12,000 x g to separate the supernatant from the cracked cells and the glass beads. The supernatant was transferred to a new tube and incubated for 5-10 minutes at room temperature. Following the incubation period, 200 µl of chloroform was added and shaken vigorously for 15-20 seconds. The tubes were once again incubated at room temperature for 2-3 minutes then centrifuged at 4° C for 15 minutes at 12,000 xg.

Following centrifuging, the different phases separated with the RNA remaining in the top aqueous phase. This layer was carefully removed and transferred to new tubes. 500 µl of isopropanol was then added and allowed to incubate for 10 minutes at room temperature. Following the incubation period, the tubes were spun at 12,000 x g for 10 minutes at 4° C. The supernatant was discarded from a whitish gel-like pellet that formed. The pellet was washed by adding 1 ml of 75% ethanol and vortexed for 15-20 seconds prior to centrifuging at 7,600 x g for 5 minutes at 4° C. This washing step was performed two times.

When washing was completed, the ethanol was removed allowing the pellet to air dry for 5-10 minutes. To break up the pellet, 50 ml of RNase free water was added and pipetted up and down, then vortexed and incubated at 55-60° C to completely dissolve the pellet.

DNA Digestion

A Turbo DNA-free kit (Ambion) was used to digest any DNA remaining in the RNA solution. A 0.1 volume of 10 X Turbo DNase buffer was added to resulting volume of RNA followed by 1 µl of Turbo DNase, then mixed gently by flicking, followed by

incubation at 37° C for 20-30 minutes. A 0.1 volume of resuspended DNase Inactivation Reagent was added, mixed by flicking and then incubated at room temperature for 2 minutes with occasional mixing. The tubes were then centrifuged at 10,000 x g for 2 minutes. The supernatant containing the RNA was transferred to new tubes.

Following the RNase purification, the sample was analyzed for concentration and purity using the Smart Spec spectrometer. If the quality of the concentration and purity of the RNA were found to be satisfactory, an RT-PCR was performed with the Actin gene serving as a control.

Primer and RNA Testing

An AccessQuick RT-PCR (Promega) was performed, in accord with the vendor's instructions, with 50ng/µl RNA samples that was collected at 24 and 120 hours from mold and yeast (totaling four samples). This procedure used a 2X Access Master Mix, 10 mM Actin primer mix, AMV reverse transcriptase and nuclease-free water in volumes per the vendor's protocol. Once the RT-PCR was completed, the samples were mixed with 6X DNA loading dye, loaded onto a 2% agarose gel and subjected to electrophoresis at 96 v/cm for 90 minutes to ascertain if a cDNA was formed. A positive result was indicated by formation of a DNA in all four lanes at 450 bp and the next procedure was carried out.

The same protocol for RT-PCR was performed with the 50ng/µl RNA samples tested in the previous experiment. This procedure also used the 2X Access Master Mix, AMV reverse transcriptase and nuclease-free water but a 10 mM *chs* primer mix

(sequences listed in Table 2) was used in place of the actin primers. Genomic DNA was also used as a control to ensure that the primers were functioning properly.

Three of the PCR tubes did not contain the AMV with the RNA and reaction mix. The tubes without AMV determined if there was any DNA contamination in the RNA samples. The remaining three tubes contained AMV along with the RNA.

Once these solutions were mixed, the PCR tubes were put through an RT-PCR program in the thermal cycler. The cycle wass as follows: 50° C forever (pre-heat), add sample, reverse transcription 30 minutes at 50° C, initial PCR activation 15 minutes at 95° C, denaturing 30 seconds at 94° °C, annealing 1 minute at 53° C, extension 1 minute at 72° C, go to step 4 (denaturing) 34 times, final extension 10 minutes at 72° C then 4° C forever. Upon the completion of the program the samples were mixed with 6X DNA loading dye and run on a 2% agarose gel. If no DNA was present in the tubes without AMV, there was no banding in the lanes on the gel for these two samples of RNA. If there was a banding pattern at the proper base pair for the two tubes with the AMV added, it was assumed that this is the expression of the *chs* provided by the primers. The tubes containing DNA always produced a band at the proper base pair for the *chs*.

This protocol was performed for all CHS primers A, B, D, E, F, G, and P to ensure proper function. A PCR using DNA was also preformed to show the functionality of the CHS primers.

Temperature Gradient

A temperature gradient PCR was performed to determine optimal temperature for *chs* expression. Temperatures ranged from 45° C to 60° C on all seven genes. A 2% agarose gel was run on each PCR to determine optimal annealing temperatures.

Experimental Cultures

Penicillium marneffei was grown and conidial collection was performed per the previously described protocol. Chitin synthase gene expression was assessed after cultures of conidia in SAB broth grew at 25°C and 37°C in shaking water baths for 24 and 120 hours.

The aforementioned protocol was followed for obtaining pellets and cracking cells. RNA was obtained using the Ribozol (Amersco) protocol followed by the Turbo DNase free protocol. An AccessQuick RT-PCR (Promega) was preformed for all genes for all growth phases using Actin as a control. The resulting PCR products were separated on a 2% agrose gel to ascertain the level of chitin synthase expression. This was performed in triplicate to ensure consistent data.

One-step RT-PCR

Template RNA, primers of interest, dNTP mix, 5X Qiagen One-step RT-PCR buffer were thawed and placed on ice. A master mix was prepared using the primers (forward and reverse), dNTP, enzyme and the buffer 5X for a total volume of 50 µl. This was mixed thoroughly and dispensed in the proper amounts into PCR tubes. RNA was added right before the PCR tubes were placed in the thermocycler. The QRANRT

program was then run. The cycle is as follows: 50° C forever (pre-heat), add sample, reverse transcription 30 minutes at 50° C, initial PCR activation 15 minutes at 95° C, denaturing 30 seconds at 94° C, annealing 1 minute at 53° C, extension 1 minute at 72° C, go to step 4 (denaturing) 34 times, final extension 10 minutes at 72° C then 4° C forever.

Table 2. CHS sequences for all seven gene primers for RT-PCR with AMV and RT-PCR without AMV experiments.

Chitin synthase gene	Sequence
chs A	F 5' – CGCTTAACAGACCCCTATGC – 3' R5' – AATGCCGGTTGGTTCATAAG – 3'
chs B	F 5' – GTCGTCGTTGGCTTAATGGT – 3' R5' – ATGGTTGCGAACGATGTGTA – 3'
chs D	F 5'– TCAGTGGTCTGTTGCTGATG – 3' R5'– CCGATTTCGCTGGTGATAAG – 3'
chs E (*csm A*)	F5' – CACATTTGACAGCCCATTTG– 3' R5'– CAACCGATGGTCGAGAATTT – 3'
chs F	F5'– GCCAATCGCTGGATTTATGT– 3' F5'– CATGGAGTTTGGTGGTGGTA– 3'
chs G	F5'– CGTTGGTTGAACGGTTCTTT – 3' R5'–AATGCCGAAAAAGATGAACG– 3'
chs P (*csm B*)	F5'– CCTTGCTTAATTCGCTCCAC– 3' R5'–TGGATAACGCGAGTGTCAAG – 3'

RT qPCR

To quantify chitin synthase gene expression, a QuantiFast Sybr Green RTqPCR (Qiagen) was performed on all seven of the chitin synthase genes in mold and yeast forms incubated for 24 and 120 hours. This protocol utilizes 2x QuantiFast SYBR Green RT-PCR Master Mix, Quantifast RT Mix, forward and reverse CHS primers and the isolated RNA. All reactions were set upon ice to avoid premature synthesis of cDNA. A

reaction mix was prepared according to the Qiagen's reaction set-up table in appropriate volumes for samples that was used. This mix was divided per the number of genes being run per plate. To each aliquot of reaction mix, the forward and reverse CHS primers were added. This was further divided into three to accommodate the RNA dilutions.

The RNA was diluted for three dilutions; the original dilution, 1:10 and a 1:100 before being added to the tubes containing the reaction mix and the primers. From each tube, 25 µl of solution was loaded, in triplicate for each reaction, into a 96 well plate. The plate was loaded into the machine and the program was run per the manufacturer's suggested protocol. The thermal profile for RTqPCR was: 55° C for 10 minutes, 95° C for 5 minutes followed by 40 repeats of 95° C for 10 seconds, 60° C for 30 seconds with real time analysis then 81 repeats of 55°C for 1 minute with melt curve analysis (temperature change), 5 with end temperature of 95° C) followed by 4°C forever.

Once the program was completed, the iQ5 software generated standard curves, melting curves and normalized gene expression. The standard curve displayed the efficiency of the reaction, the coefficient of the determination (R^2), the slope of the line and the y-intercept. To utilize data, the efficiency should be close to 100% and the R^2 should be as close as possible to 1.00. Standard curve/C_t results can be displayed in a spreadsheet which will display the well, fluorophore, sample type, identifier, replicate number, threshold cycle, starting quantity and statistics for replicate groups.

Melting curves for each gene were determined by assigning a beginning and ending temperature to each peak then calculates an area beneath the curve. It is important to note that each gene should have one melting point per condition. The curve will rise until the temperature reaches that gene's melting temperature, being the highest point of

the peak, then decline. Different melting curves point to DNA contamination, primer-dimers or artifacts.

Reference genes were used to normalize data by measuring their level of expression as a normalizing factor. This was calculated by using the quantities of the reference gene to normalize the values of the gene of interest. This eliminated the need to use other methods to normalize data. These calculations take in to consideration differences in loading and cell number variations in the sample. The software utilized a modification of the equation introduced by Livak et al (1995) to evaluate normalized expression referred to as the ddC_t or $\Delta\Delta C_t$ value.

Table 3 CHS sequences for all seven genes' primers for RT qPCR.

Chitin synthase gene	Sequence
XM_002145210 [*csm A* (*chsE*)]	F 5'-CCC CTC CCA TAC ACA ATC TTC – 3' R 5' – CAG ACA AAT CAA AGC CTG Cg -3'
XM_002145211 [*csmB*]	F 5' - GTA TCG TTG ATC CCC AGT TAG G - 3' R 5'- GGA GTA GAT AGT GGT AAG ATA GAA AGG -3'
XM_002148485 [short *chsA*]	F 5' - TTA TAC AGACAA TCG CAC TCC C - 3' R 5' - GTT GAT TCC TTC GAC CGA GTA C -3'
XM_ 002148034 [*chsB*]	F 5' - GCG TTG CAA TTT ATC CTC TCG -3' R 5' - TCC ACC GCC TTT CAG TTT ATC -3'
XM_002146248 [*chsD*]	F 5' - GTG TCA TCG CGG TTT TCA TC -3' R 5' - TTA ACG AGA GCC CAG GAA AAG-3'
XM_002149542 [*chsF*]	F 5' - AGC CgA GGG TGA ATT TGA TAG -3' R 5' - GGT AGG CAT ATG GCG CTG -3'
XM_002153543 [*chsG*]	F 5' - CTA CC TGT TCC TAG TGC TAT TC -3' R 5' – CGT AAG TTG TAA CCG TTG TGC -3'

Cloning and Plasmid Isolation

To verify the identity of the RT qPCR products, the resulting amplification products were cloned using the TA cloning kit (Invitron) in accord with the manufacturer's instructions. The resulting clones were used to inoculate LB broth with AMP and subsequently incubated in a shaking water bath at 37° C overnight. Cells were collected from the inoculated broth by placing 1.5 ml of the broth in a centrifuge tube for 15 minutes at 15,000 RPM. The supernatant was then carefully poured off. The plasmids from these cell pellets were isolated using the QIAcube (Qiagen) in conjunction with a DNA mini prep kit (Qiagen). The purity and concentration of the isolated plasmids were determined spectrophotometrically.

Plasmid sequencing

The amount of plasmid needed for sequencing was calculated using the table provided by Beckman Coulter. In PCR tubes, 8 µl of Quickstart Reaction Mix (Beckman Coulter), 2 µl of primer at 1.6 pmol/ µl (one tube forward and one tube reverse), determined amount of plasmid and nuclease-free water to make a total volume of 20 µl were added. These tubes were placed in the thermocycler and the SEQ-DTCS program was run. The thermal profile for SEQ-DTCS was: 93° C for 20 seconds, 50° C for 20 seconds, and 60° C for 4 minutes repeating from step one 29 times followed by 4°C forever. Sequencing products were subsequently purified in accord with the vendor's instructions and then sequenced using a Beckman Coulter 3000XL sequencing system.

Chapter III

Results

Primer Testing.

PCR using PMF4 DNA was performed on the CHS primers to ensure they were working correctly. Both the RT-PCR primers and the QRT-PCR primers were tested. All CHS primers generated the expected product sizes based on the designed length as displayed by Table 2 and 3, figure 1. It was noted that *chsA* primer (XM_002148485) displayed some double banding.

Experimental Rationale.

Penicillium marneffei cultures incubated at 25° C and 37°C in SAB at 24 hours displayed unique morphological differences when observed microscopically. These differences became more distinct as incubation continued. At 25° C hyphae were thinner, longer and had multiple branches when compared to those incubated at 37° C which were short and broad. At 96 hours, the hyphae incubated at 37° C phase transitioned to yeast cells through a process called arthroconidiogenesis.

These morphological differences represent critical developmental processes which can be observed in early growth phases and later during phase transition. It was reasoned that early and late growth would be ideal to observe the chitin synthase expression where growth begins and later when phase transition occurs. Hence, the focus of observation of chitin synthase expression was during mold and yeast development

after 24 and 120 hours. Therefore, samples observed in this study will be referred to as mold 1 day, yeast 1 day, mold 5 day and yeast 5 day.

RNA was extracted from mold and yeast at these two growth points. It was analyzed to ensure it had a purity of 1.8 to 2.0. All RNA samples used throughout this study, whether using the Ribozol protocol or the Qiacube, were at 1.8 or higher. It was also tested to ensure there was no DNA contamination.

RT-PCR with AMV.

RT-PCR was performed with CHS primers designed from 270 -460 bp with Avian myeoblastosis virus, which is an RNA dependent polymerase. DNA was also used as a control. All seven of the CHS genes were used along with Actin as a control, with samples collected from 24 and 120 hour cultures of mold and yeast. All samples displayed no DNA contamination and interestingly the first hint of differential expression was observed.

Temperature Gradient

To ensure the proper annealing temperatures were being utilized for each *chs*, a temperature gradient was performed. The temperatures started at 45° C and ended at 60°C. This procedure was performed for each gene to verify an annealing temperature suitable for that gene. A majority of the genes displayed proficient annealing temperature within 3 degrees lower than the melting points of the forward and reverse primers.

RT-PCR.

RT-PCR with all the genes at all the conditions was run to observe if there were any differential expressions of chitin synthase. These protocols were performed with Actin as a control and then analyzed on a 2% gel.

Chitin synthase A displayed expression in mold and yeast at both one day and five days. *ChsB* displayed expression only in early phase mold one day and *chsD* displayed no expression at any of the time points. *chsE* (*csmA*) displayed expression in mold and yeast for both time frames. *chsF* appeared to display relatively small amounts in early mold and late yeast phases. Based on the 2% agarose gel, *chsG* seems to be displayed at all time frames for both yeast and mold but at various intensities. Finally, *chsP* (*csmB*) which is considered to have overlapping functions with *csmA*, appeared to show expression only in the early mold phase.

Table 4: All CHS primers' respective base pair based on the designed length for RT-PCR.

GENE	Size of DNA Product	Size of cDNA Product
chsA	353	318
chsB	492	397
chsD	464	362
chsE (*csmA*)	603	459
chsF	333	276
chsP (*csmB*)	488	423

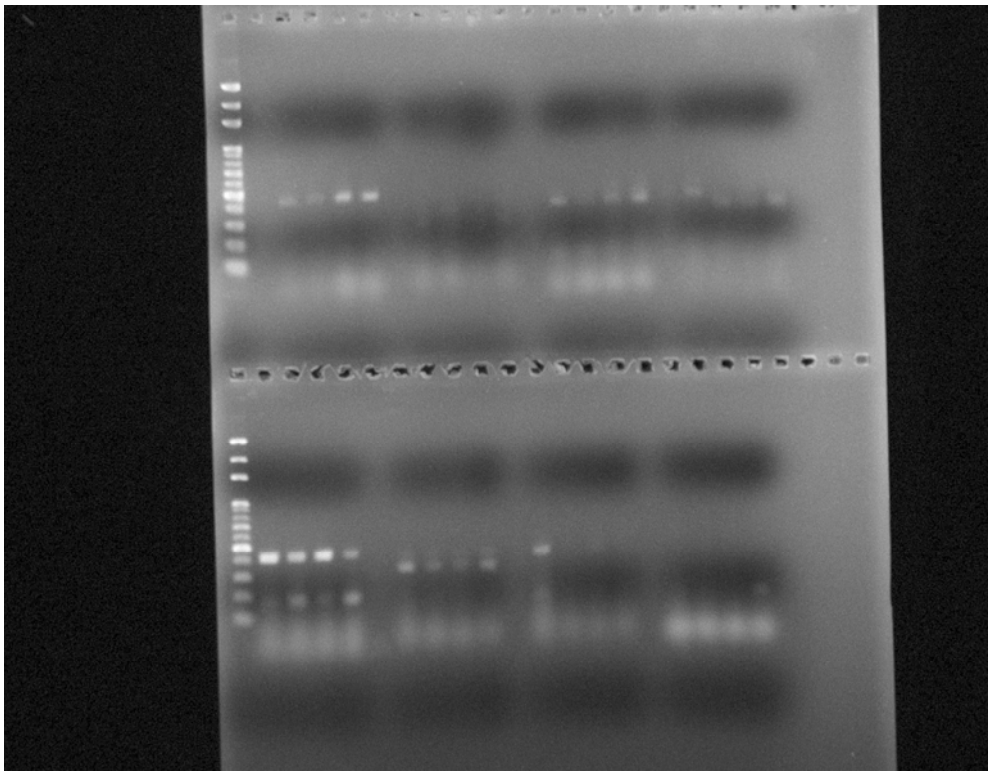


Figure 1: RT-PCR products for Actin and all seven chitin synthase genes. Starting with the bottom left, lane 1 ladder; lanes 2-5 Actin M1, Y1, M5, Y5; lane 6 empty; lanes 7-10 *chsA* M1, Y1, M5, Y5; lane 11, empty; lanes 12-15, *chsB* M1, Y1, M5, Y5; lane 16, empty; lanes 17-20, *chsD* M1, Y1, M5, Y5. Upper left, lane 1, ladder; lane 2 empty; lane 3-6, *chsE* (*csmA*) M1, Y1, M5, Y5; lane 7, empty; lanes 8-11, *chsF* M1, Y1, M5, Y5; lane 12, empty; lanes 13-16, chs G M1, Y1, M5, Y5; lane 17, empty; lanes 18-21, *chsP* (*csmB*) M1, Y1, M5, Y5.

RT qPCR

Reverse transcriptase quantitative PCR was performed on all seven chitin synthase genes using the SYBR Green I and the iQ^{TM}5 Optical System (BIO RAD). The iQ^{TM}5 software was able to analyze standard curves, melting curves and normalized gene expression. It utilized equations derived from the like of Pfeffl and Livak to produce identical results. Each gene was analyzed using RNA from mold and yeast phases at 24 and 120 hours. Actin was used as a control for each gene and for each condition.

Amplification charts were analyzed to determine if the increase of quantification was at least 3 cycles in range with one another. Melt curve charts determined the melting point of the RT qPCR products then melt curves were evaluated for double peaks. For any given gene, only one melting point temperature should be displayed; one gene, one peak. If any well displayed more than one peak it was eliminated from the data. This was carried out to ensure the quantification analyzed was from only one gene. Standard curves were also evaluated to determine efficiencies. This was carried out for all RTqPCRs for all seven of the genes. It is to be noted that not all aforementioned data is displayed or described herein. However, all results were consistent with data shown.

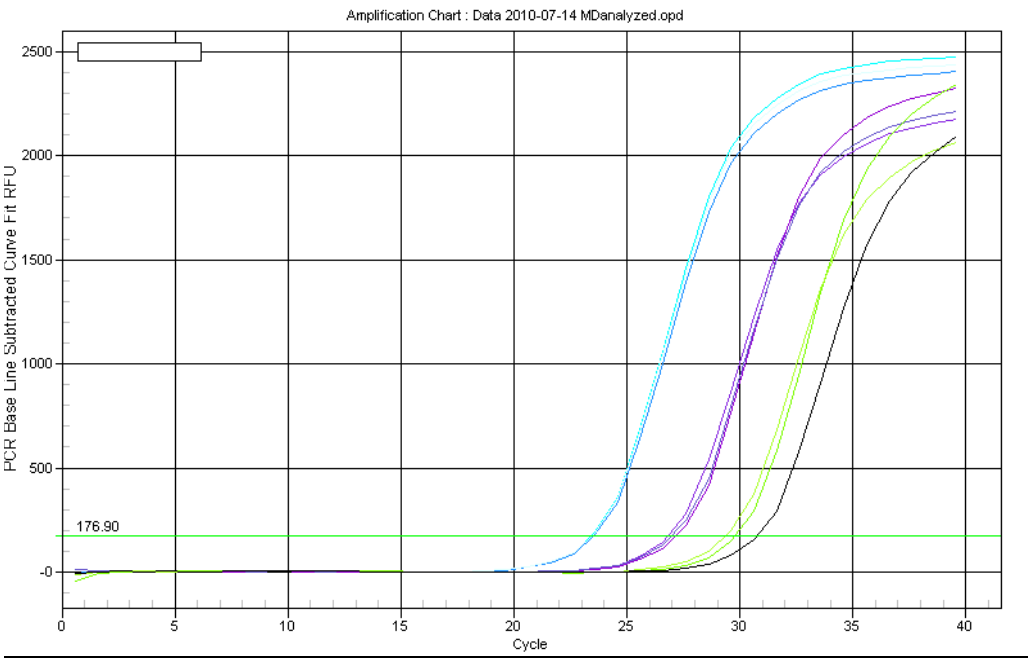


Figure 2: Amplification chart showing amplifications are within three cycles of each other

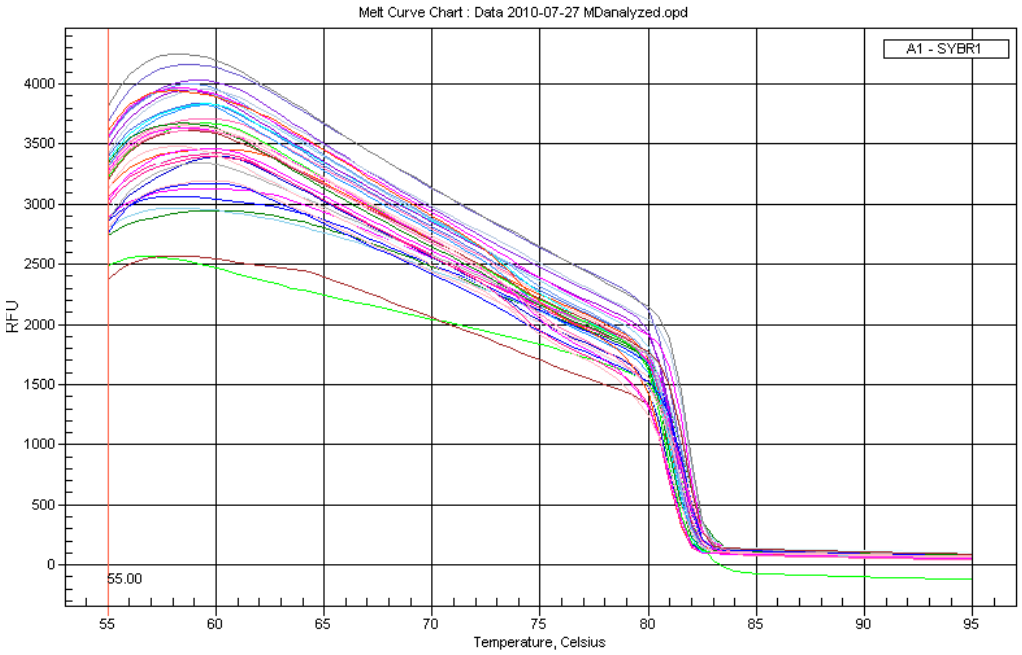


Figure 3: Melt curve chart for *chsA* showing melting point of gene of interest

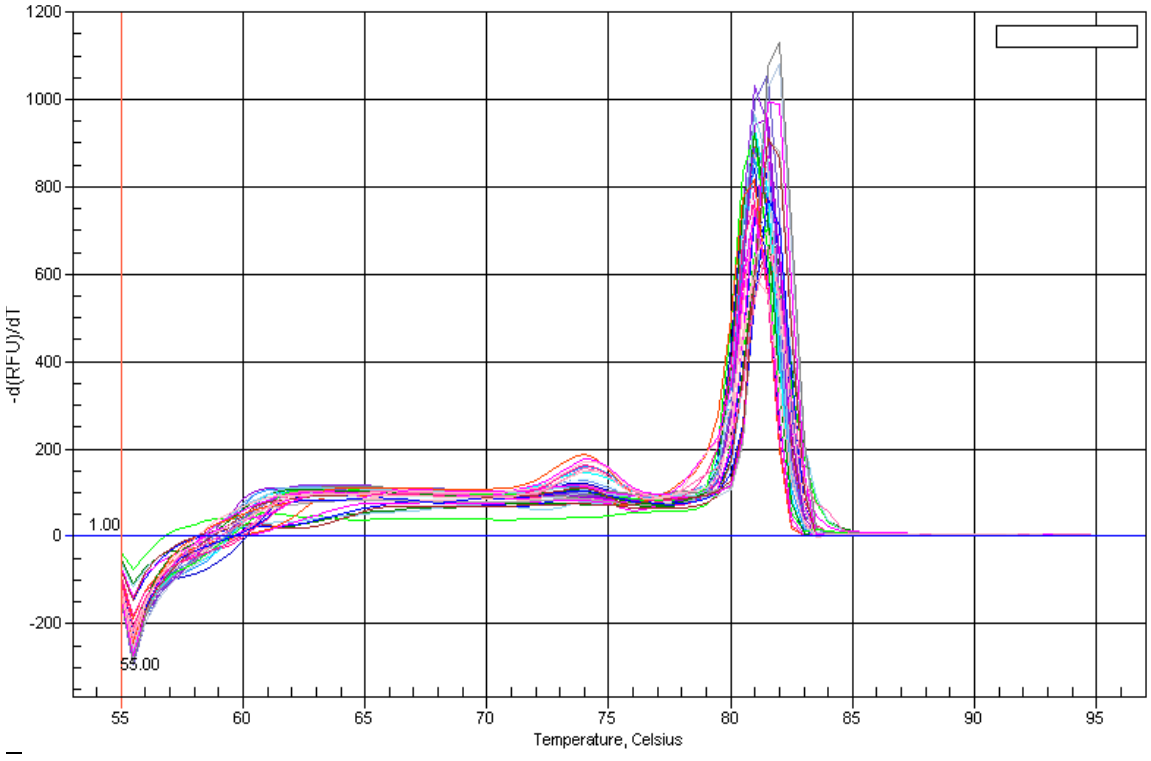


Figure 4: Melt peak chart for *chsA* and Actin displaying one TM for all conditions.

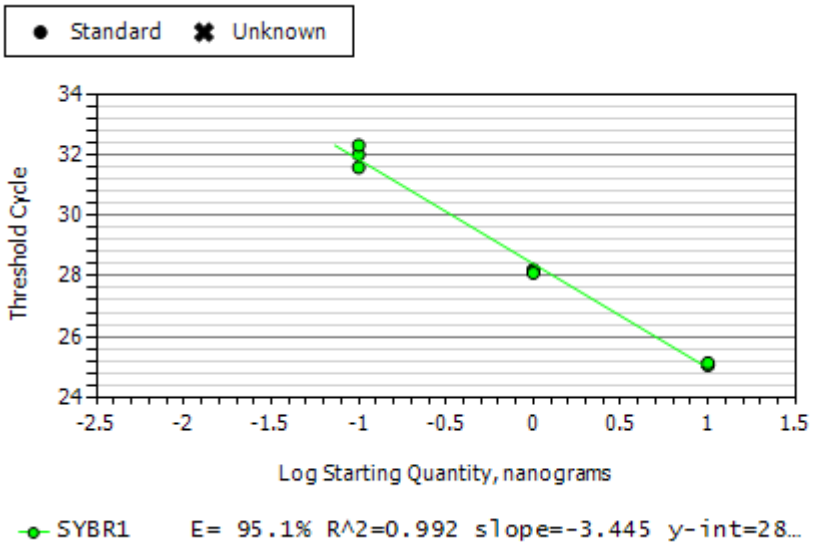


Figure 5: Standard curve for *chsA* showing efficiency at 95.1% and R^2 at 0.992

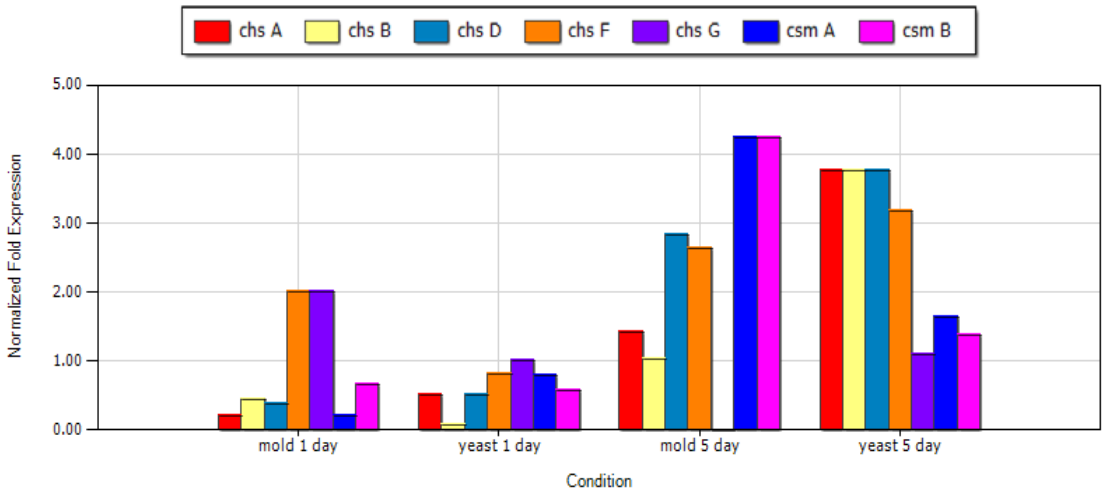
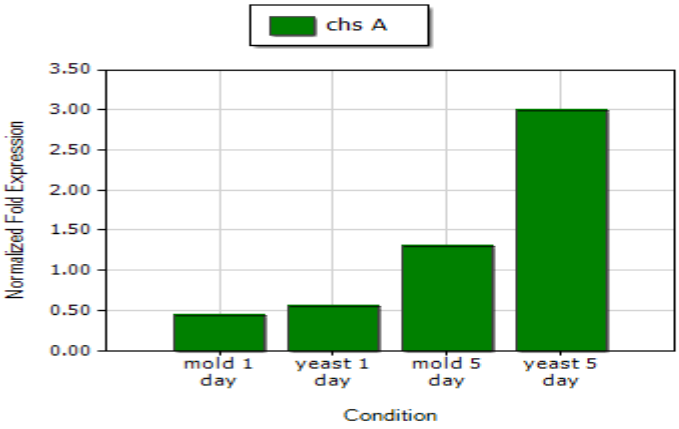
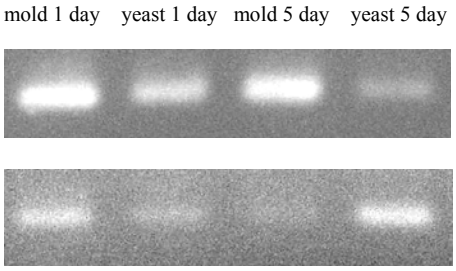


Figure 6: RT qPCR normalized fold expression results for all chs genes for all growth conditions.

chsA

Normalized fold expression was graphed for the quantification of *chsA*. It appeared that mold at five days was expressed over twice as much as mold at one day. Based on the graph, it appeared that yeast at five was also expressed more than the yeast at one day. This yielded about a five times greater amplification.



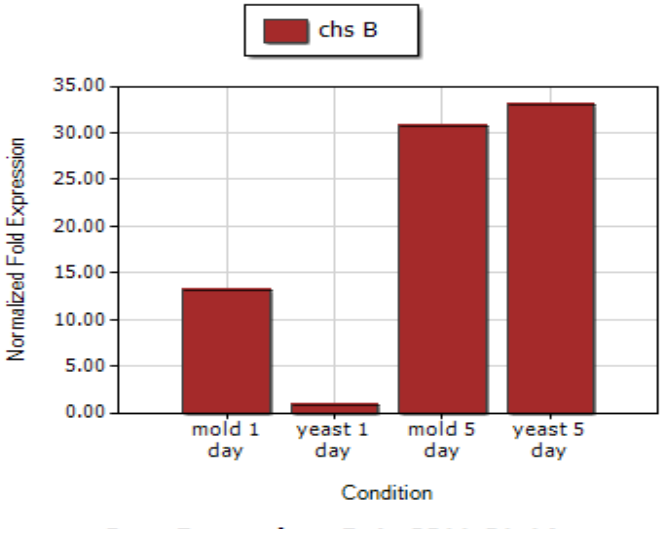


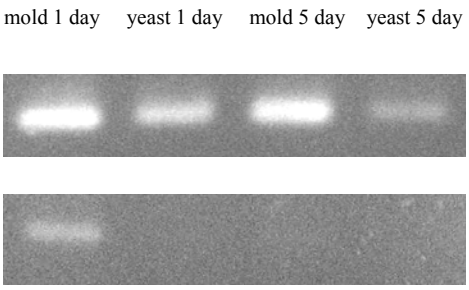
RT-PCR Results for Actin (top) and chs A (bottom) mold 1 day, yeast 1 day, mold 5 day, yeast 5 day

Figure 7: Normalized fold expression for *chsA* for mold and yeast at one and five days and RT-PCR

chsB

Normalized fold expression for *chsB* displayed far less expression when relative to zero. It appears that late stage mold and yeast are expressed at a greater amount than the early stage mold and yeast. When comparing the expression levels between mold and yeast, mold is expressed at higher rate in both phases. It is interesting to note that the expression in later phase mold is significantly higher than the yeast when comparing the expression in the early phase mold and yeast.



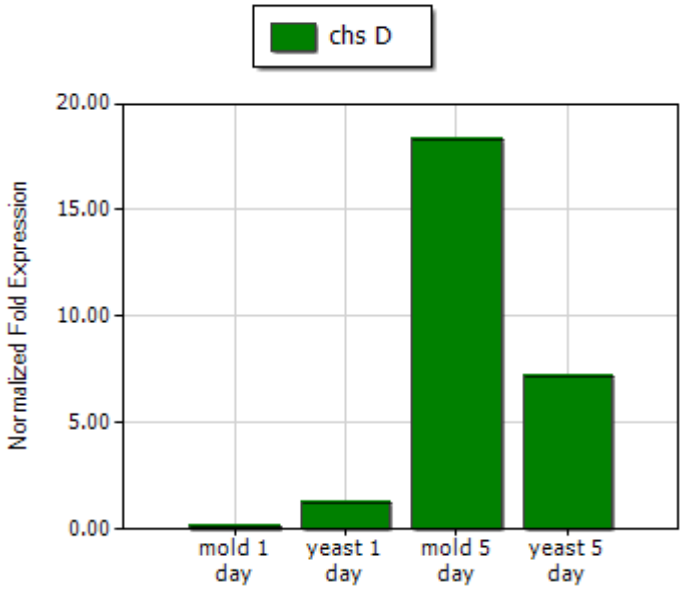


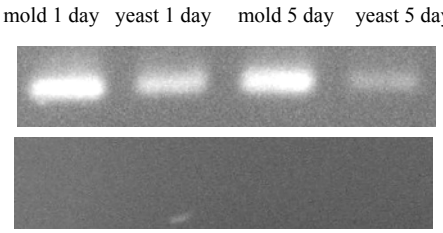
RT-PCR results for Actin (top) and chs B(bottom) at mold 1day, mold 5 day, yeast 1 day and yeast 5 day

Figure 8: Normalized fold expression for *chsB* for mold and yeast at one and five days and RT-PCR expression.

chsD

When expression levels of *chsD* were normalized to the expression of the Actin control it appears that mold at one day is expressed far less than that of mold five day. The yeast samples also follow the pattern of expression in mold; however, yeast five day isn't expressed at the level of mold five day.



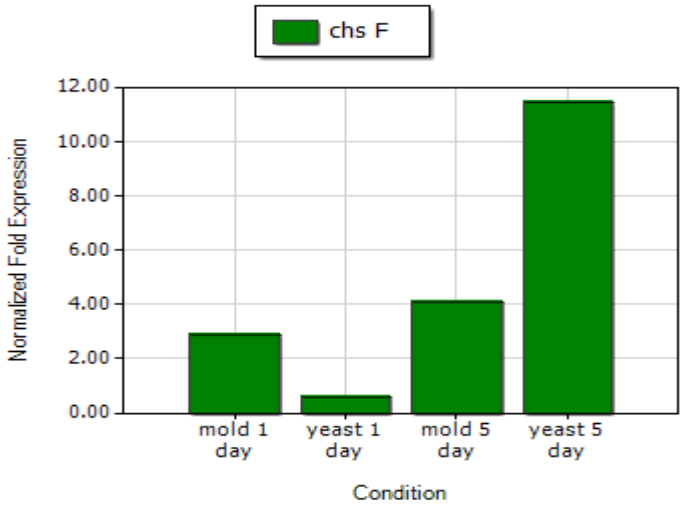


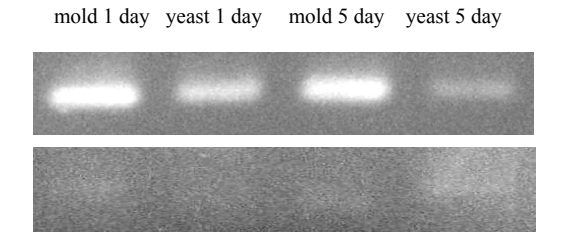
RT-PCR results for Actin (top) and chs D (bottom) mold 1day, yeast 1 day, mold 5 day, yeast 5 day

Figure 9: Normalized fold expression for *chsD* for mold and yeast at one and five days.

chsF

Normalized fold expression displayed the greatest amount of expression of *chsF* at yeast in the late phase. Mold one day and five day show small increase of expression when compared to one another whereas the two yeast phase showed great differences between expressions. It was noted that the mold in the early phase was expressed at higher rate than the yeast yet the differences reversed in the late phase conditions. The yeast displaying almost three times the amount of the mold.



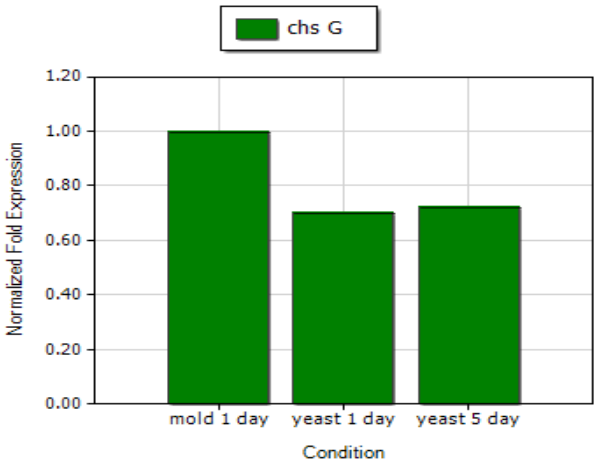


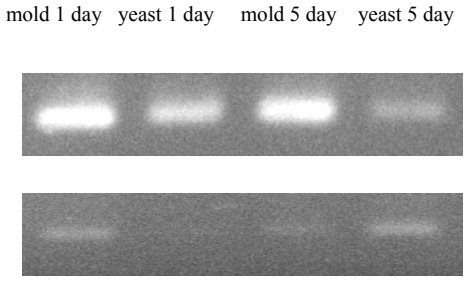
RT-PCR results for Actin (top) and chs F (bottom) mold 1 day, yeast 1 day, mold 5 day, yeast 5 day

Figure 10: Normalized fold expression for *chsF* for mold and yeast at one and five days.

chsG

Normalized fold expression for *chsG* yielded mold in the early phase to display the greatest expression. Yeast one day and five day displayed slight differences in expression yet yeast five day was a bit greater. The differences overall between the conditions was also slight but as mentioned, the mold one day did displayed the greater of the three. It is important to note that the mold late phase was not displayed due to the fact that RNA for that phase was depleted at the time of the run.



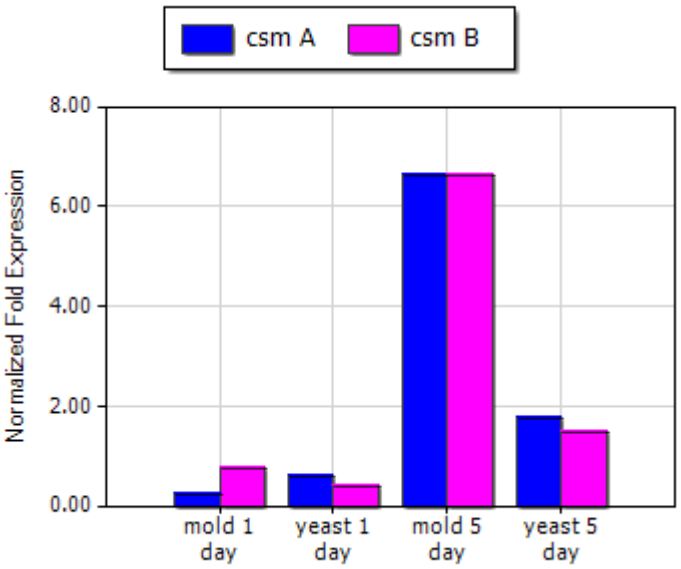


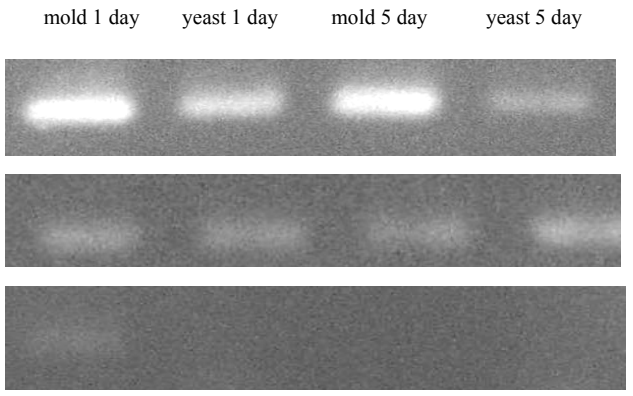
RT-PCR results for Actin (top) and chs G (bottom) mold 1 day, yeast 1 day, mold 5 day, yeast 5 day

Figure 11: Normalized fold expression for *chsG* for mold one day and yeast one and five day and RT-PCR

csmA and csmB

Normalized fold expression levels were performed together for *csmA* and *csmB* due to their proposed compensatory functions. It is believed that the two genes may possess compensatory functions, when on is turn off the other shuts down. Therefore, it was in the interest of this study to run the expression levels together. In mold one day, *csmA* is expressed less than *csmB* whereas in yeast one day the opposite occurs in expression. In mold five day, the expression levels appear to be identical and far greater than mold one day. The late phase yeast appears to show the same expression pattern as in yeast one day, with csmA being larger, yet total expression of both is at a higher level than the one day culture. The patterns do not suggest that one gene is shutting off while the other is turned on.





Top: Actin mold 1 day, yeast 1 day, mold 5 day, yeast 5 day
Middle: csm A mold 1 day, yeast 1 day, mold 5 day, yeast 5 day
Bottom: csm B mold 1 day, yeast 1 day, mold 5 day, yeast 5 day

Figure 12: Normalized fold expression for csm A and csm B for mold and yeast at one and five days and RT-PCR expression.

Cloning

In melt peak charts of *chsA*, double peaks were being displayed consistently. The condition yeast 5 day seemed to display a different melting peak than the other conditions. In standard RT qPCR results, this data would have been eliminated. However, based on research on *chsA*, it appeared to have two transcription sites. If this indeed was the case, two different forms of *chsA* would have been displayed based on sequencing. Therefore, sequencing of chs A RT qPCR results seemed worthwhile.

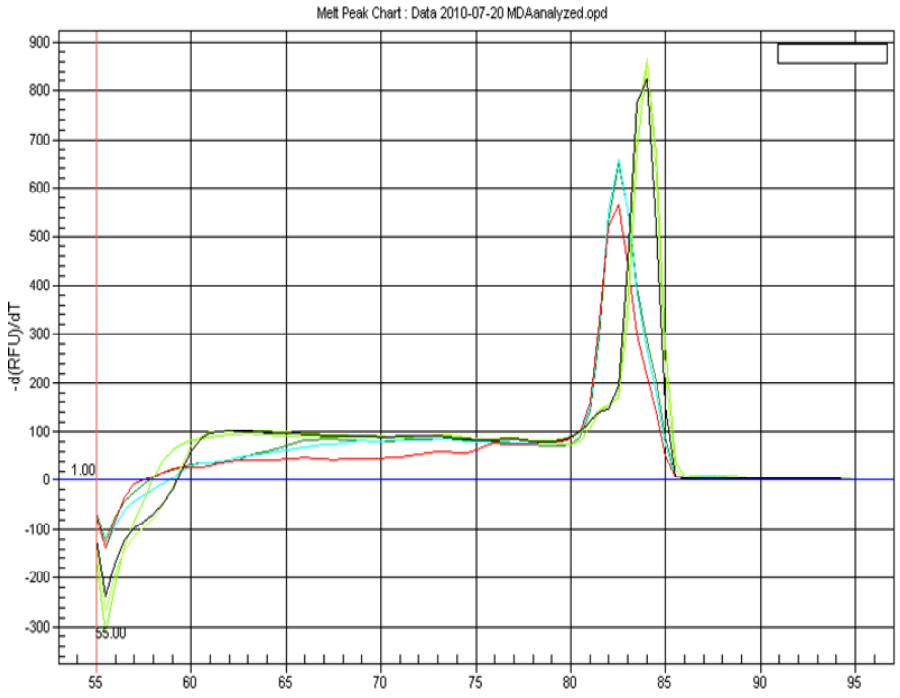


Figure 13: Melt peak chart for chs a displaying two different melt peaks. Chs A yeast 5 day displayed higher melt peak (84 ^{o}C) while other conditions were consistently at 82 ^{o}C in majority of the RTqPCR reactions run.

Sequencing results from the products of RT qPCR in *chsA* mold 1 day, yeast 1 day, mold 5 day, yeast 5 day all indicated they were indeed *chsA*. When sequencing results were run through BLAST (NCBI), two possible matches were displayed; long *chsA* and short *chsA* (see appendix Figure 20).

Figure 14 displays the transcription sites for the short and the long versions of *chsA* which appeared in the BLAST results. The primers used for the RT qPCR were that of the short *chsA*, therefore the rational for the two BLAST results is as follows. The short version's sequence of *chsA* is located within the sequence of the long version therefore both versions will always be displayed in BLAST.

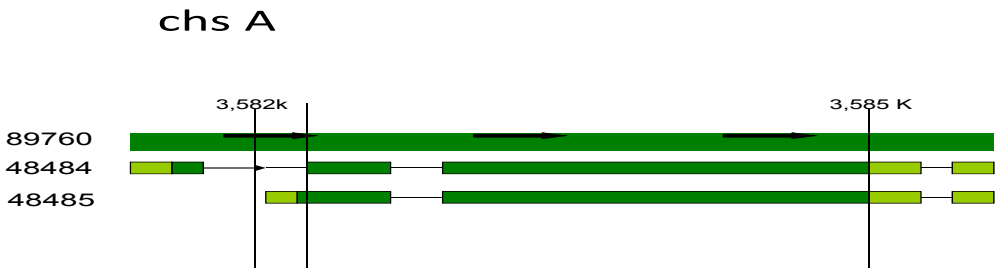


Figure 14: Transcription sites for long and short *chsA*.

To further investigate whether the two forms of *chsA* were being expressed, primers were designed for both the long and the short *chsA*. A forward primer was designed for 48484 (84 F primer sequence: ccaccatgccttcctacaat) to begin shortly after its first intron as well as for 48585 (85 F primer sequence: tgatgcagcgcttaacagac) along with a reverse primer (*chsA* R primer sequence: gatactgtcgtcgccatgtg) that was located within both versions. This was designed to test whether expression in the mold and yeast phases

was only in one version or if they were displayed in both. Preliminary results displayed some expression with both primer sets; however, further testing is needed to give conclusive results.

Chapter IV

Discussion

The results from the RT-PCR and RT qPCR for all seven chitin synthase genes support the rationale of the hypothesis of differential gene expression in the mold and yeast forms of *Penicillium marneffei*. Based on the expression, the correlation of the proposed function of each gene at the respected developmental phase was theorized. The multiplicity of CHS enzymes gives way to the possibility that they may have redundant cell wall synthesis roles. Therefore, determining a cut and dry function for these genes may not be determined, but rather a suggested function may be derived. Also it is important to remember that purported functions are merely given from gene orthologues. No deletion research has been performed with *P. marneffei*.

Chitin synthase gene expression in *P. marneffei* was displayed using RT-PCR and then quantified using RT qPCR. Differential gene expression was present in RT-PCR for all chitin synthase genes which verified the hypothesis. The hypothesis was further validated using RT qPCR to analyze the relative quantification which displayed, in graph form, the differential expression. Determination of the gene expression in 24 and 120 hour cultures can aid to verify purported functions.

ChsA displayed expression at all growth phases according to RT-PCR. Upon further analysis with RT qPCR, expression appears to be equal in early phase mold and yeast. However, late phase expression is far greater; up to five times as much in the yeast sample. Proposed functions of *chsA*, based on gene orthologues with *A. fumigatus* and

A. nidulans, suggest it may be involved in hyphal growth. As literature suggests, chitin synthases may possess multiple functions therefore, late phase expression can also point to the data found with *chsA*'s gene orthologue in *Aspergillus nidulans* which suggest that *chsA* contributes to mature ascospore cell walls and could play a relevant role in the maturation of ascospores (Lee et al, 2004). The results shown in Figure 7 display greater *chsA* expression in 120 hour cultures which would support the literature's proposed functions in mature ascospores.

The expression in *chsB* was also displayed at a greater rate in late phase conditions. Research suggests that *chsB* is expressed mainly in condia, phialides, matulea, vesicles and stalks in sexual differentiation which would be developed in late phase mold (Lee et al, 2004). It is also suggested that *chsB* may have redundant functions with *chsA* involving hyphal growth and conidial development A (Horiuchi, 2009). These findings agree with the pattern of expression from RT qPCR. Mold cultures at 24 hours are expressed greater than yeast, however, expression in 120 hours cultures of both mold and yeast display the greatest expression (Figure 8).

Little is written on the function of the gene orthologues to *chsD* in *marneffei*. Expression in RT-PCR seemed to be non-existent yet RT qPCR displayed expression which validates RT qPCR as being far more sensitive in reading gene expression. Expression seemed to be most significant in early mold phase and equal in the other growth phases. Some literature suggests that *chsD* is involved required for conidiation therefore expression in early mold would be relevant. However, some research points to the function of *chsD* to be non-important to the survival or synthesis of chitin in the cell

wall. Perhaps it merely serves as a support system when introduced stresses endanger the functions of other genes which serve the same function.

RT qPCR expression in *chsF* mold early phase showed a greater amount of expression than in the yeast early phase. However this trend reverses in the late phase expression. It is also interesting to note that research has determined the functions of *chsF* overlap with *chsB*. Some suggested overlapping functions are in conidia formation. When comparing the expression (Figures 8 and fig. 10) of the two, expression is almost identical. In *chsB*, late phase mold is expressed at a greater rate than in *chsF,* however, the pattern of the two genes' expression overall is similar. Purported functions in *chsF* suggest it has control of the rigidity of the walls of germinating conidia, subapical regions of the hyhae and conidiophores vesicles yet that it may be non-essential in the asexual cycle (Specht, 1996; Horiuchi, 2009). Research also indicated that the function of *chsF* increased when the expression of *chsG* was repressed once again alluding to the notion that chs have multiple functions (Horiuchi, 2009).

The results of RT qPCR for *chsG* in 24 and 120 hour cultures (Figure 11) reveal relatively close expression, as did the expression in RT-PCR. The mold culture appeared slightly higher than the expression of yeasts, which were practically identical. The purported functions could explain the consistency in the expression as it is suggested that it is involved in all aspects of asexual development. It is also suggested that it contributes to normal hyphal growth and organization by creating a scaffold for other wall polymers to be deposited (Lee et al, 2004). Therefore the need for this gene to be expressed at all time points in both mold and yeast is apparent.

Literature on *csmA* and *csmB* suggested that they have compensatory in hyphal tip growth. It is proposed that if one gene is performing this function then the other will down-regulate. RT-PCR data displays expression of both genes in mold early phase yet *csmB* expression is non-existent in yeast early phase and mold and yeast late phase. This would suggest that the idea of compensatory functions is valid. Both genes initially are turned on then *csmA* continues to be expressed while *csmB* appears to down-regulate during certain growth phases. RT qPCR results for early mold phase expression in *csmA* were less than *csmB*, yet in yeast one day the results are opposite with *csmB* being expressed more. Mold five day *csmA* and *csmB* expression levels appear to be at the same level. However, yeast five day *csmA* and *csmB* follow the same expression pattern as yeast one day only at a higher level. These results seem to suggest some compensatory functions could be occurring; when one gene is expressed more, the other is expressed less. The existence of both genes also may suggest that while one is functioning at a greater level, the other may serve an additional function or provide a support system.

CONCLUSION

The pathogenic fungi *P. marneffei* is the only *Penicillium* species that displays a thermally dependent dimorphism. Pathogenicity of *P. marneffei* is associated with this dimorphism. The dimorphism presumably reflects changes in the cell wall structure which is mainly composed of chitin.

Studies to determine the genes that control the dimorphism are inconclusive. Therefore, gaining a better understanding to the temporal and spatial expression of chs genes in *P. marneffei* would be of great use. Such findings in the the expression of chitn

genes during different growing phases in mold and yeast would then be compared with purported functions.

To do this, RT-PCR was run on all seven chitin synthase genes to display qualitative expression. The hypothesis was verified when differential expression was displayed throughout the genes at the different time phases. Further analysis using RT qPCR gave insight to the relative amount of expression per gene.

The purposed functions were compared to the expression patterns in each gene. Logical conclusions were drawn based on these comparisons. It should be noted that functions of the genes were based on the orthologues from *A. fumigatus* and *A. nidulans* which are similar species yet may have slightly different functions. Also, chitin synthase genes have so many overlapping and multiple functions under different conditions that the functions attached to the genes are educated conclusions at best.

Therefore it is important to obtain knowledge of chitin gene expression in mold or yeast in different time frames to help better understand cell wall changes. Expression under normal growth conditions would provide a base line for much needed research. Researchers could perform deletions of chitin synthase genes independently or in combination to ascertain the morphological and expression changes. This could lead to discoveries pertaining to morphogenesis, virulence and create a pathway for novel drugs that target the cell wall.

Chapter V

Appendix

Melt peak, melt curve and standard curve charts for *chsB*

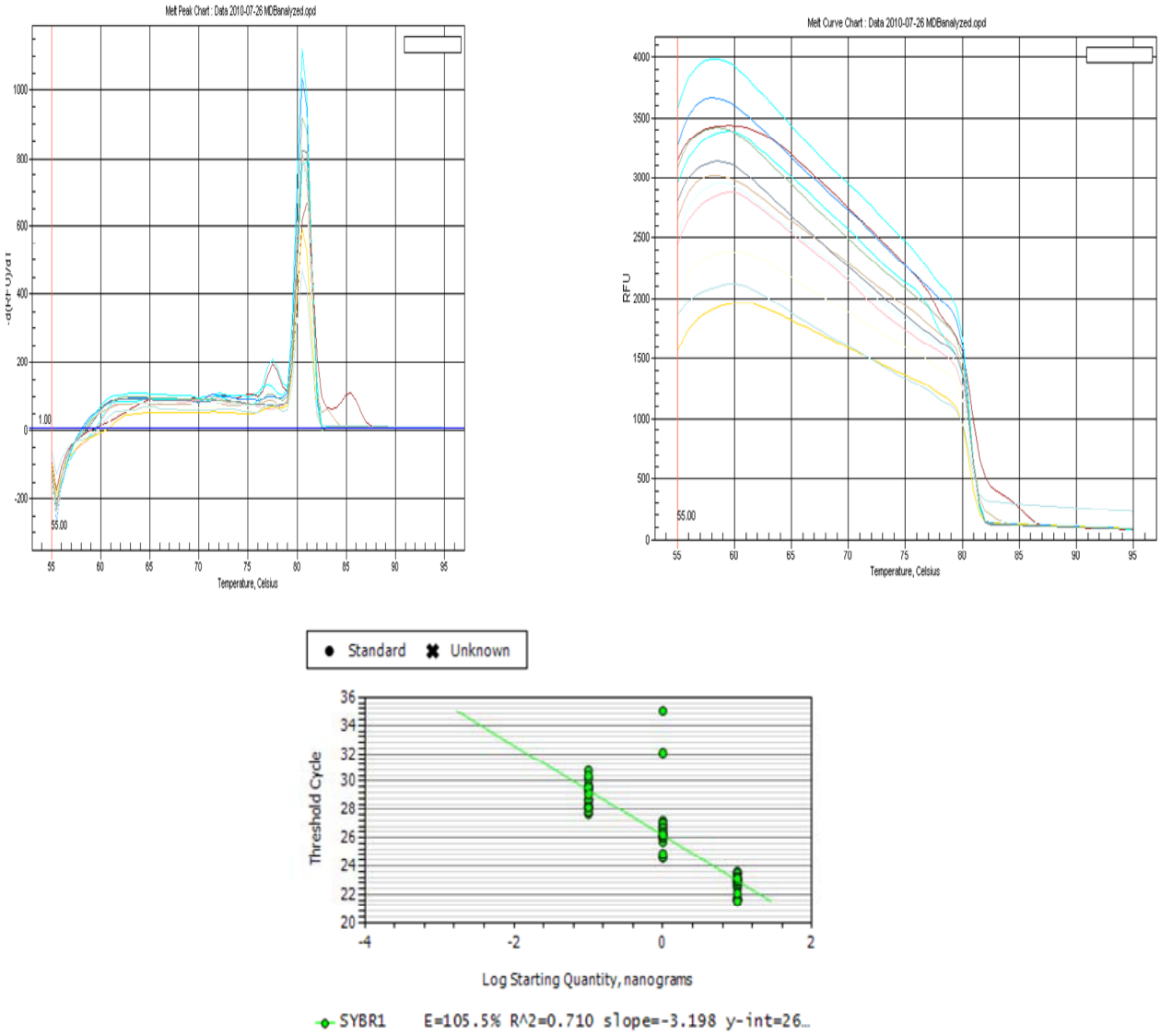


Figure 15: Melt peak and melt curve charts for chsB all show one gene of interest being amplified. Standard curve chart displays high efficiency.

Melt peak, melt curve and standard curve charts for *chsD*

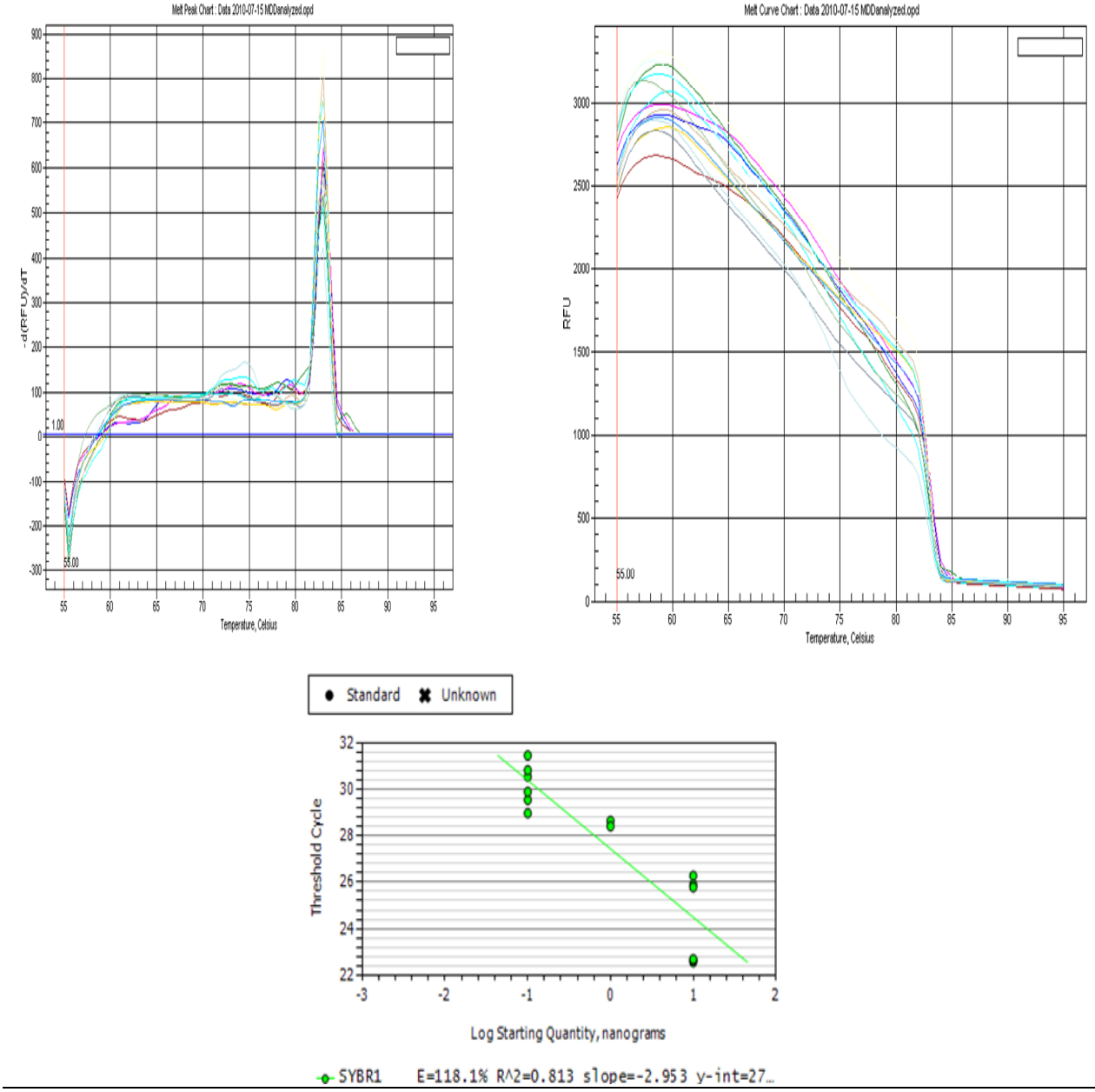


Figure 16: Melt peak and melt curve charts for *chsD* all show one gene of interest being amplified. Standard curve chart displays high efficiency.

Melt peak, melt curve and standard curve charts for *chsF*

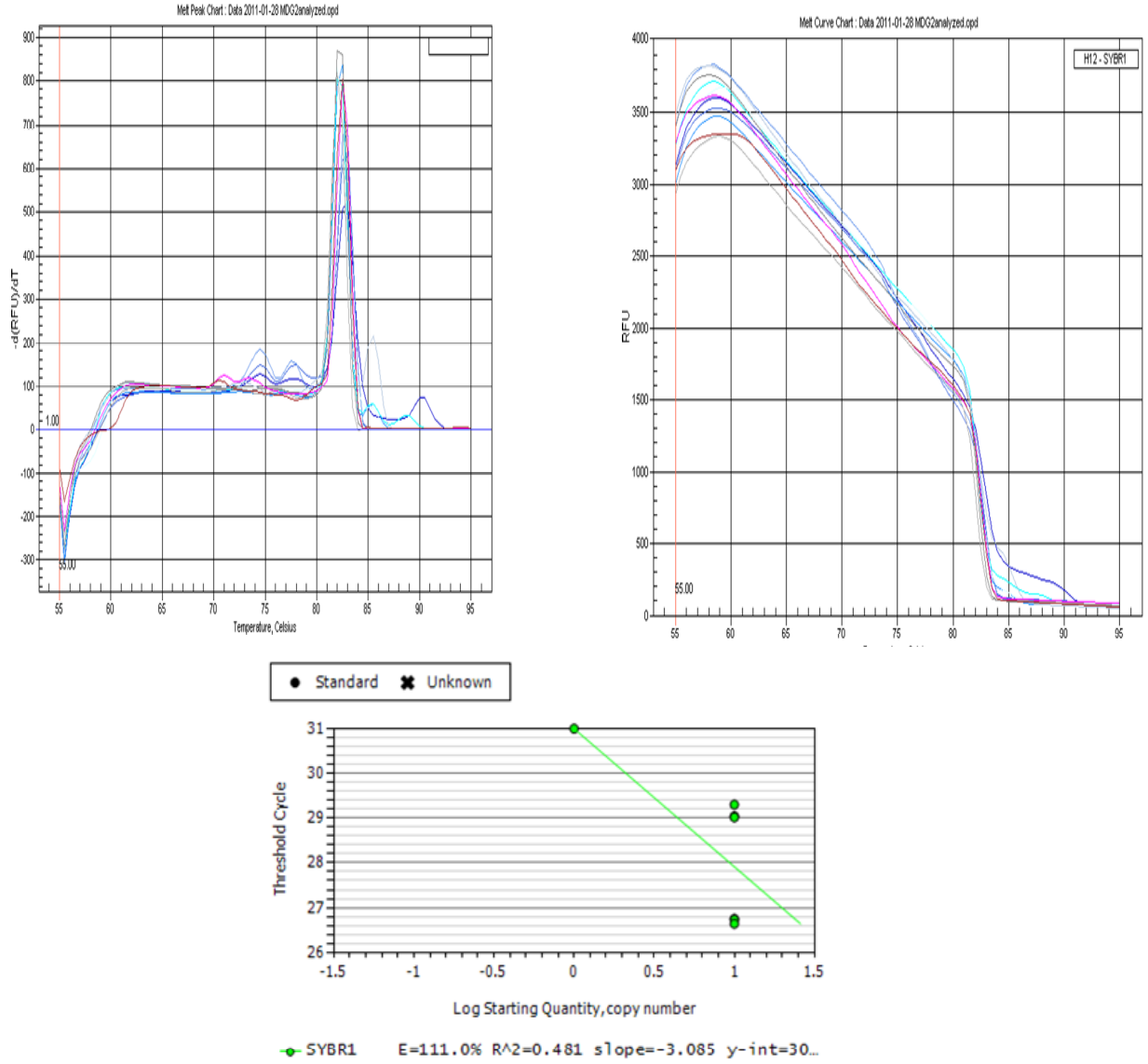


Figure 17: Melt peak and melt curve charts for *chsF* all show one gene of interest being amplified. Standard curve chart displays high efficiency.

Melt peak, melt curve and standard curve charts for *chsG*

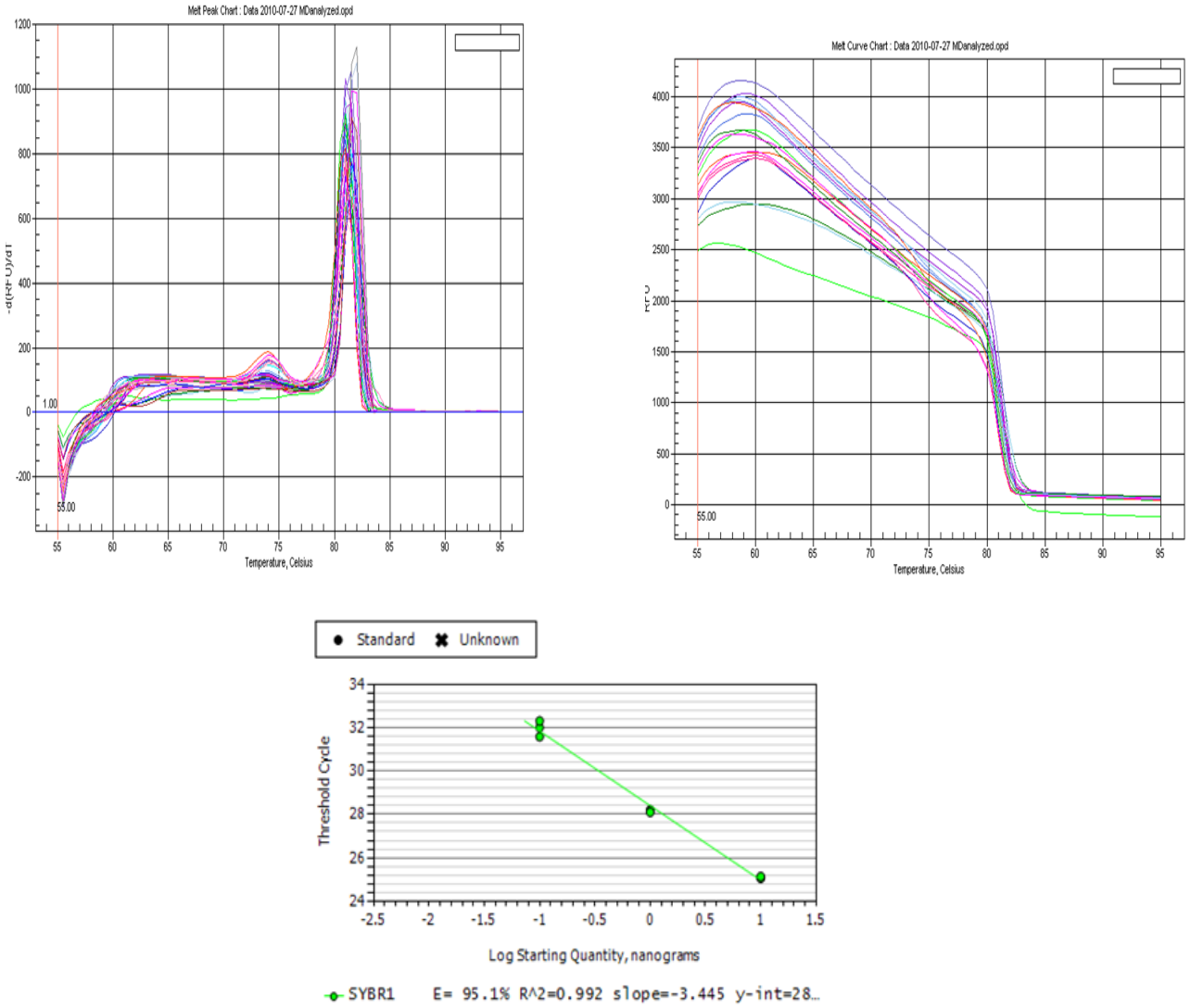


Figure 18: Melt peak and melt curve charts for *chsG* all show one gene of interest being amplified. Standard curve chart displays high efficiency.

Melt peak, melt curve and standard curve charts for *csmA* and *csmB*

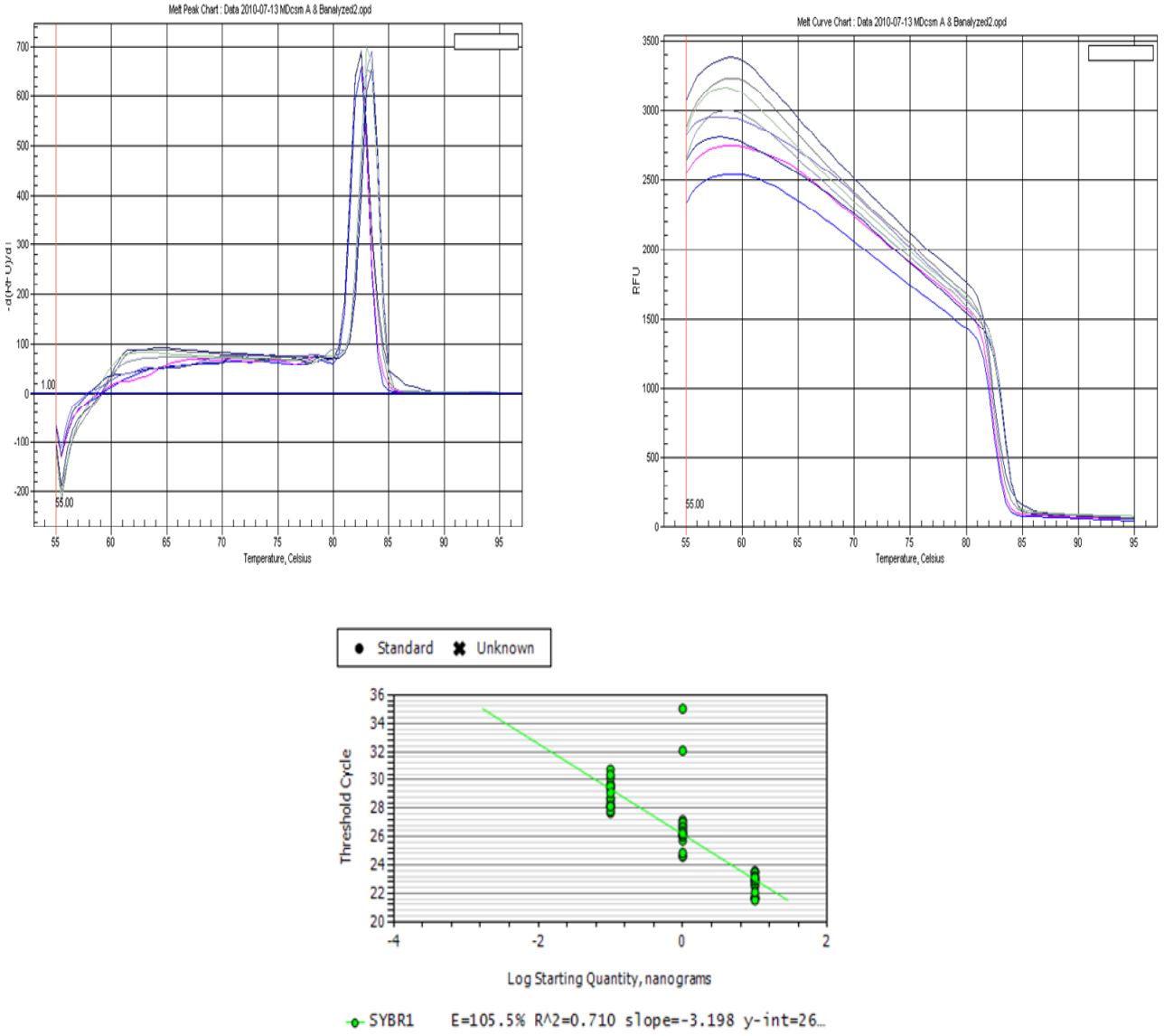


Figure 19: Melt peak and melt curve charts for *csmA* and *csmB* all show one gene of interest being amplified. Standard curve chart displays high efficiency.

TTTATACAGACAATACGCACTACCCTGCCTCCGGGGCGGCCATAACAGCCATATCATACATGATAATCAGAGTTTC
ATAGCCACTGCACGATGCACCTCTACAGATCATCTGCTATGACCAGCCTACGTACTCGGTCGCAAGGAATCACACA
A

>ref|XM_002148485.1|

Penicillium marneffei ATCC 18224 chitin synthase A, mRNA
Length=3354

GENE ID: 7026103 PMAA_089760 | chitin synthase A
[Penicillium marneffei ATCC 18224]
Score = 156 bits (172), Expect = 2e-38
Identities = 134/152 (89%), Gaps = 14/152 (9%)
Strand=Plus/Plus

```
Query  2    TTATACAGACAATACGCACTACCCTGCCTCCGGGGCGGCCATAACAGCCATATCATACAT  61
            |||||||||||||  ||||||  ||||  ||  ||||||||||||  ||||||||||||  ||||
Sbjct  144  TTATACAGACAAT-CGCACT-CCCTCCC--CGGGGCGGCCATTACAGCCATATCA-ACAT  198
Query  62   GATAATCAGAGTTTCATAGCCACTGCACGATGCACCTCTACAGATCATCTGCTATGACCA  121
            |  ||  |||||||||  | ||||||||  |||||  ||||||||||||||||||||  |||||
Sbjct  199  G--AAACAGAGTTTC--AACCACTGCA-GATGC-CCTCTACAGATCATCTGCTA-GACCA  251
Query  122  GCCTACGTACTCGGTCGCAAGGAATCACACAA  153
            ||||||||||||||||  |||||||||  ||||
Sbjct  252  GCCTACGTACTCGGTCG-AAGGAATCA-ACAA  281
```

>ref|XM_002148484.1|

Penicillium marneffei ATCC 18224 chitin synthase A, mRNA
Length=3313

GENE ID: 7026103 PMAA_089760 | chitin synthase A
[Penicillium marneffei ATCC 18224]
Score = 156 bits (172), Expect = 2e-38
Identities = 134/152 (89%), Gaps = 14/152 (9%)
Strand=Plus/Plus

```
Query  2    TTATACAGACAATACGCACTACCCTGCCTCCGGGGCGGCCATAACAGCCATATCATACAT  61
            |||||||||||||  ||||||  ||||  ||  ||||||||||||  ||||||||||||  ||||
```

Figure 19: Graph displaying differential expressions for all *chs* in *P. marneffei* in 24 and 120 hour cultures.

```
Sbjct  211  GCCTACGTACTCGGTCG-AAGGAATCA-ACAA  240
```

Figure 20: Sequencing and BLAST (NCBI) results from cloning experiment of *chsA* yeast 5 day.

Chapter VI

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