Phenotypic Characterization and Gene Expression Analyses of a *Penicillium marneffei* Septin Mutant

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Daniel E. Kennedy, II

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Daniel E. Kennedy, II

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Signature:

Daniel E. Kennedy, II, Student Date

Approvals:

Dr. Chester Cooper, Thesis Advisor Date

Dr. Gary Walker, Committee Member Date

Dr. Jonathan Caguiat, Committee Member Date

Dr. David Asch, Committee Member Date

Peter J. Kasvinsky, Dean of School of Graduate Studies and Research Date

ABSTRACT

Penicillium marneffei is the only known thermally dimorphic species of the *Penicillium* genus, growing as a filamentous mold at 25˚C and adopting yeast morphology at 37˚C. Endemic to Southeast Asia, there is no known reservoir for *P. marneffei*. Nonetheless, the evolutionary adaptation of phase transition has allowed *P. marneffei* to become a highly aggressive, opportunistic pathogen. Since the emergence of AIDS, *P. marneffei* infections have become increasingly common due to its increased virulence when invading immunocompromised individuals with most cases being reported in Thailand.

Phase transition in *P. marneffei* is tightly regulated at the transcriptional level. Gene expression in *P. marneffei* is regulated when subjected to higher or lower temperatures. These thermally dependent, phase specific regulatory mechanisms are of current interest, as understanding such mechanisms will potentially provide targets for chemotherapeutic agents in treating *P. marneffei* infections in the future. Presently, an *Agrobacterium tumefaciens*-mediated transformation (AMT)-generated mutant in septin function is being studied in order to establish its role in phase transition and normal vegetative growth. Preliminary results indicate that the disruption mutation in the *aspC* gene caused aberrant morphological characteristics. In addition, *aspC* expression in the *aspC* mutant was greater than that in the wild-type strain.

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Chapter I

Introduction

Fungi are highly active participants in most of the world's ecosystems. The roles of most fungi are limited to degrading organic matter in their respective environments. Some fungi exist as symbionts with plants and other organisms, while others are harmful, opportunistic pathogens. While most *Penicillium* species appear to be quite common and are not involved in infection of humans, several species from the *Penicillium* genus, including *Penicillium marneffei*, have been documented as causative agents of a condition called penicilliosis (Cooper and Haycocks 2000). However, *P. marneffei* differs from other *Penicillium* species in that it is the only thermally dimorphic species of the genus, undergoing phase transition, also termed the dimorphic switch, after entering the host organism's body. *Penicillium marneffei* typically grows as a mold at temperatures between 8.0˚C and 31.0˚C (Cao et al. 2007). After internalization of *P. marneffei*, however, phase transition occurs, with concomitant changes in morphology. In addition, up- or down- regulation of various genes can be observed, as *P. marneffei* further develops as a yeast. Penicilliosis due to *P. marneffei*, while extremely rare in those with healthy immune systems, has proven terminal without treatment in those with and without suppressed immune systems. Penicilliosis, which usually manifests itself as a systemic yeast infection in its latter stages, has also become the third most common AIDS-defining illness (Supparatpinyo et al. 1994). With penicilliosis due to *P. marneffei* on the rise, the *P. marneffei* genome, including genes coding for proteins involved in cell wall construction and maintenance, phase transition, and those proteins that allow *P. marneffei* to survive in host organism phagocytic cells, has recently become a subject of

interest in a search for potential targets of chemotherapeutic agents. However, in order for such genes or their corresponding proteins to be targeted, *P. marneffei*'s genome should be further annotated.

History and Epidemiology of *P. marneffei*

Penicillium marneffei was initially isolated from a bamboo rat grown in captivity for experimental infections at the Pasteur Institute of Indochina, Dalat, South Vietnam in 1956 (Capponi et al. 1956). Despite experiments designed to reveal the native reservoir of *P. marneffei*, results have not yielded a definitive origin. Rather, it seems the conditions related to infection are host-specific factors along with environmental risk factors; age and an agricultural occupation are the most common independent factors (Chariyalerstak et al. 1997). Specifically, exposure to soil during the nearly seven months-long rainy season of Southeast Asia, from May through October, appear to have the highest corollary effect of any risk factor (Chariyalerstak et al. 1996). However, similar experiments designed to uncover the correlation of infection in native bamboo rats to infection in native human hosts have also failed to elucidate any known vectors of transmission (Cooper 1998; Chariyalerstak et al. 1996). These findings point to *P. marneffei* infections existing as a result of sapronosis, rather than zoonosis (Vanittanakom et al. 2006). In other words, an environmental reservoir should exist, rather than direct rat-human interactions being responsible for infection. The trail of infection is further convoluted when taking into account that many experiments attempting to isolate *P. marneffei* from soil samples near areas home to infected human and rat hosts alike have failed to yield positive results (Joshi et al. 2003; Vanittanakom et al. 1995). Vanittanakom et al. demonstrated *P. marneffei* could survive several weeks in

sterilized soil, while *P. marneffei*'s survival was reduced to only a few days grown in non-sterilized soil. Such findings indicate that competing fungi decrease the abundance of *P. marneffei,* thus limiting the potential for a native reservoir in Thailand.

The first documented case of the disease was reported by Professor Gabriel Segretain in 1959, who also identified the fungus as a new *Penicillium* species (Segretain 1959). It was during a laboratory experiment that he accidentally inoculated himself with a fungus-contaminated needle and later developed a nodule at the site of infection, lymphangitis, and lymphadenopathy. The first naturally occurring case was described in 1973 in an American minister with Hodgkin's disease who had lived in Southeast Asia (DiSalvo et al. 1973). Subsequently, mostly due to the rise of HIV/AIDS infection, increased travel to southeast Asia and non-sterile intravenous drug use engaged in by some of those who do, cases of penicilliosis due to *P. marneffei* infection have been documented in Cambodia (Bailloud et al. 2002), China (Liao et al. 2002), Hong Kong (Chang et al. 1998; Ko 1994; Tsang et al.1991; Tsui 1992; Wong et al. 1998), India (Ranjana et al. 2002), Malaysia (Rokiah et al. 1995), Taiwan (Chang et al. 1995; Chiang et al. 1998; Hsueh et al. 2000; Hung et al. 1998; Liu et al. 1994), Thailand (Sathapatayavongs et al. 1989), Vietnam (Hien et al. 2001; Huynh et al. 2003), Australia (Heath et al. 1995; Jones and See 1992), Belgium (Depraetere et al. 1998), France (Grise et al. 1997; Hilmarsdottir et al. 1994; Hilmarsodottir et al. 1993; Valeyrie et al. 1999), Germany (Rimek et al. 1999; Sobottka et al. 1996), Japan (Mohri et al. 2000; Tsunemi et al. 2003), Sweden (Julander and Petrini 1997), Switzerland (Borradori et al. 1994; Garbino et al. 2001; Kronauer et al. 1993), The Netherlands (Hulshof et al. 1990; Kok et

al. 1994), the United Kingdom (Bateman et al. 2002; McShane et al.1998; Peto et al. 1988; Vilar et al. 2000), and the United States (Nord et al. 1998).

Medical Significance

Although most *P. marneffei* related cases of penicilliosis have occurred in Southeast Asia, the source of *P. marneffei* remains largely unknown. Globally, *P. marneffei* has proven itself highly pathogenic in AIDS patients, becoming the third most common AIDS-defining illness and yielding the third highest rate of opportunistic pathogenesis among studied AIDS patients (Cooper and Haycocks 2000). Penicilliosis in both HIV-positive and HIV-negative individuals results in death if left untreated. However, penicilliosis in HIV-positive individuals requires a much more lengthy treatment protocol than in their HIV-negative counterparts, due to the commonality of relapse after cessation of antifungal therapy. Penicilliosis in HIV-positive individuals usually occurs in the advanced stages of HIV infection due to the depletion of CD4+ T cells, and those infected typically exhibit symptoms including fever, anemia, leukopenia, weight loss, diarrhea, cough, massive hepatosplenomegaly, and generalized lymphadenopathy (Noritomi et al. 2005; Desakorn et al. 2002). Affected patients also present characteristic, multiple, umbilicated skin lesions on the face and torso. Treatment of penicilliosis due to *P. marneffei* involves lengthy rounds of oral and intravenous antifungal agents and, in some cases, surgery (Cooper and Haycocks 2000). The pathogenesis of *P. marneffei* makes treatment of infections highly difficult, increasing the urgency in understanding this opportunistic pathogen.

General Biology, Dimorphism, and Pathogenicity

As with other fungi, *P. marneffei*'s ability to regulate cell wall construction is critical for growth. Because cell wall construction is so important to the survival of *P. marneffei*, it should come as no surprise that the process is tightly regulated. Cell wall synthesis involves simple molecules arranged into conglomerate complexes and enzymes, the latter being responsible for arranging those molecules in such intricate patterns. Although *P. marneffei's* cell wall composition has yet to be completely characterized, a general pattern of cell wall construction for each phylum of fungi has been established. Like many studied models of the Phylum Ascomycota, *P. marneffei's* cell wall is presumed to be composed of two polysaccharides, namely chitin and glucans, and mannoproteins (Applegarth and Bozoian 1967; Bacon et al. 1960; Domer et al. 1967; Kanetsuna et al. 1969). These macromolecules can be further divided into structural and matrix components, the latter serving to cross-link the former. Chitin and β-(1→3) - and β-(1→6)-glucans provide structural support in the form of microfibrils, while mannoproteins and α (1→3)-glucan are found in the matrix of the cell wall. Because the mold phase morphology manifests itself as apical growth at the hyphal tip, also termed the extension zone, those polysaccharides that provide the cell wall rigidity, chitin and β-glucans, are abundant in the cell wall behind the extension zone but exist in relatively low levels in the extension zone cell wall itself. More precisely, chitin chains are synthesized at the apex by chitin synthase and subsequently inserted into the cell wall as the apex continues to grow. Similarly, β-(1→3)-glucan chains are synthesized at the apex by glucan synthase, inserted into the wall, and further linked to one another by β-(1→6)-linked side chains. Structural polysaccharides aggregate laterally into increasingly

complex crystalline microfibrils at higher levels farther from the extension zone (Pancaldi et al. 1984). Increasing the structural polysaccharide content in the hyphal cell wall allows the fungus to maintain rigidity (i.e., protection) in the non-expanding portion of its cell wall, while growth continues at the tip.

Cell wall synthetic enzymes, chitin synthase and glucan synthase, are most likely transported to the apical cell wall in vesicles (Deacon 2006). Once embedded in the cell wall chitin synthase is likely activated via protease. Cytosolic N-acetylglucosamine (GlcNAc) is bound to a sugar nucleotide substrate, uridine triphosphate (UTP), yielding UDP-GlcNAc, which is then added to an elongating chitin chain, in a reaction cleaving UDP, by chitin synthase. Comparatively, glucan synthase uses another sugar nucleotide substrate, cyotosolic UDP-glucose, to synthesize glucan chains. In contrast with chitin synthase, however, glucan synthase is a dimeric G-protein. Though, in both cases of microfibrillar formation, chitin and glucan chains are extruded through to the outer surface of the membrane and into the cell wall, where they provide structural integrity.

Perhaps the most puzzling trait of *P. marneffei* is its mode of pathogenesis. *Penicillium marneffei* grows as mold and yeast forms at 25˚C and 37˚C, respectively, and is the only known thermally dimorphic species of the *Penicillium* genus (Canovas and Andrianopoulos 2007). At 25˚C, *P. marneffei* displays asexual reproduction, during which a filamentous body, the hypha, exhibits periodic branching and apical growth. From these filamentous bodies arise conidiophores, composed of specialized cells (Andrianopoulos 2002). Secondary stalks called rama may also be produced from basal, vegetative, hyphal cells. Rama then differentiate to produce the sterigmata cell types: metulae and phialides. Distinctive from vegetative, apically-growing, multinucleate

hyphal cells, metulae and phialides are produced via budding division and are uninucleate. In turn, the metula then buds one or more phialides. Phialides then generate conidia in a basipetal manner, producing chains of conidia. However, should the nutritional requirements (e.g., amount or type of carbon source) not be met, rama may be absent. Consequently, sterigmata bud directly from the hyphal cell, or shorter, unbranched stalks may produce conidiating stergimata cells. In addition, carbon-limiting conditions also cause conidiophore production earlier than would occur otherwise. Once conidia have been freed from the phialide, they disperse and may germinate once finding suitable growth conditions. These conidia grow isotropically at first, then produce a germ tube. The latter marks the beginning of polarized hyphal growth (Canovas and Andrianopoulos 2007). Asexual development in *P. marneffei* is controlled by a number of regulatory and signaling genes, some of which have been characterized. Highly similar homologues of these genes have been well characterized in *Aspergillus nidulans*, including *brlA* and *abaA*, with the former activating the latter (Adams et al. 1988). In *P. marneffei*, *brlA* is believed to encode a C_2H_2 zinc finger protein, which consequently activates *abaA* (Adams et al. 1998). The *abaA* gene product is an ATTS/TEA protein (Andrianopoulos and Timberlake 1994; Burglin 1991). ATTS/TEA proteins in *P. marneffei* are DNA- domain transcriptional regulators suspected of playing roles in cell-cycle events, conidiation, and hyphal-yeast development (Borneman et al. 2000). AbaA, in turn, causes vegetative hyphae to begin conidiophore production (Borneman et al. 2000) and removes conidiation from the influence of other inductive signals (Cooper and Vanittanakom 2008). *abaA* deletion strains of *P. marneffei* were shown to be deficient in conidiation, producing conidiophore stalks possessing abnormal sterigmata cells

incapable of producing conidia (Borneman et al. 2000). *abaA*, as well as *brlA*, is further regulated by a member of the APSES family of transcription factors, *stuA*, which is responsible for sterigmata, metula, and phialide development during conidiation (Borneman et al. 2002). *stuA* contains a basic helix-loop-helix (bHLH) DNA binding motif. Experimentation has shown that, while the *stuA* transcript was undetectable during vegetative hyphal growth, it was highly induced once the conidiation program had begun. *stuA* deletion strains exhibited conidiophores lacking the characteristic sterigmata cells. However, conidia continued to be produced basipetally directly from the tips of conidiophore stalks, showing *stuA* to be essential for sterigmata cell differentiation but not for conidiation. Adding to the complexity of the gene regulation pathway during asexual development are *gasA*, *cflA*, and *cflB*. The Gα subunit of GasA inhibits asexual development by repressing *brlA*, maintaining a state of vegetative growth (Zuber et al. 2002). In contrast, *P. marneffei* Rho GTPases Cdc42 and Rac homologues, *cflA* and *cflB*, respectively, carry out overlapping functions affecting growth at both 25˚C and 37˚C (Boyce et al. 2001; Boyce et al. 2003). *cflA* is required for germination of conidia at 25˚C and 37˚C, polarized growth and division of hyphae at 25˚C, activating the p21 activated kinases (PAKs) *pakA* and *pakB* (Richman et al. 1999; Lamson et al. 2002; Benton et al. 1997; Gladfelter et al.2004; Peter et al. 1996), and polarized growth of yeast cells at 37˚C. Like *cflA*, *cflB* is also responsible for cellular polarization and division during hyphal growth, but, unlike *cflA*, *cflB* also plays a role in conidiophore cell differentiation to the extent that *cflB* deletion strains result in yeast-like morphology and inappropriate yeast cell production at 25˚C (Boyce et al. 2009; Boyce et al. 2003). Similarly, CflB has experimentally been shown to colocalize with actin in the tips of

vegetative hyphal cells and at sites of septation, and, while CflB is not required for localization of cortical spots and actin at nascent septation sites, it is required for actin localization at the hyphal tip (Boyce et al. 2003). However, *cflB* deletion in similar experiments did not affect yeast cell production, with cellular polarization occurring relatively normally at 37˚C. The two aforementioned *P. marneffei* PAKs are associated with a multitude of signaling and morphogenic activities, which play a role in the more severe pathological effects caused by *P. marneffei*. For instance, in *pakA* deletion strains, conidial germination failed to occur after conidia had been phagocytized by macrophages, limiting the ability of intramacrophage hyphal growth and subsequent arthroconidiation, strongly suggesting that at least systemic penicilliosis due to *P. marneffei* requires *pakA* expression. Related experimentation has also shown *pakA* to be essential for conidial germination and correct yeast cell morphogenesis at 37˚C, and is required for conidial germination *in vivo* (Boyce and Andrianopoulos 2007). Experimental results have also revealed *pakB* to be an integral cell polarity gene during infection (*in vivo*), but not *in vitro* (Boyce et al. 2009). Macrophages infected with wild-type *P. marneffei* conidia contained yeast cells, whereas macrophages infected with *pakB* deletion strains contained highly branched, septate, hyphal cells but no yeast cells. This data suggests that PakB is essential for pathogenicity by *P. marneffei*.

Those afflicted with penicilliosis have most likely inhaled the conidia. Once a conidium reaches an alveolus, its ability to recognize fibronectin (Vanittanakom et al. 2006) and bind laminin in the bronchoalveolar epithelium via a scialic acid-specific lectin is suspected to aid adherence (Rongrungruang and Levitz 1999; Boyce et al. 2009). The conidium is to be phagocytized by monocyte-derived, alveolar macrophages, thus

initiating infection and potentially causing systemic penicilliosis in the host. Once the conidia have been subjected to an environmental temperature of 32˚C to 37˚C (Cao et al. 2007), the dimorphic switch occurs. *Penicillium marneffei* morphology and physiology at 37˚C differ markedly from that at 25˚C. In a process termed arthroconidiation, cellular and nuclear division become coupled (Borneman et al. 2000), and the hyphae form an additional internal septum at already existing septa, creating a double septum and a means to free the arthroconidia (Chan and Chow 1990; Garrison and Boyd 1973). Once the arthroconidia have been freed from the hyphal body they enter the yeast phase, during which cells grow in a less polarized manner than germinating conidia nor grow apically as hyphae do, but divide via fission (Chan and Chow 1990; Garrison and Boyd 1973; Pitt 1979; Segretain 1959). The arthroconidia represent the pathogenic growth phase of *P. marneffei*. However, subjecting the yeast phase cells to 25˚C reverses the dimorphic switch, causing arthroconidia to polarize and begin apical growth; cellular and nuclear growth will uncouple; and mold phase growth will be resumed (Cooper and Haycocks 2000; Canovas and Andrianopoulos 2007). It is the dimorphic switch that makes *P. marneffei* unique among the *Penicillia*.

As with asexual development, yeast cell morphogenesis in *P. marneffei* is tightly controlled. The previously described hyphal-yeast dimorphic switch is most notably characterized by the coupling of cellular and nuclear division, septation of cells leading to cell separation, and division of subsequent yeast cells via fission (Andrianopoulos 2002). Unlike during asexual development, *stuA* does not play a role dimorphic switching in *P. marneffei* (Borneman et al. 2001; Borneman et al. 2002). Conversely, the inhibitory effects of TupA, a transcription factor that is also homologous to genes associated with

the regulation of asexual development in other filamentous fungi, that normally repress the activation of *abaA*, and thus the dimorphic switch, at 25˚C, are overcome by the exposure to the increased temperature cue of 37˚C (Todd et al. 2003). Consequently, *abaA* is expressed, and AbaA, the ATTS protein, causes coupling of cellular and nuclear division. Similarly, the effects of CflA, PakB, and PakA aid in both conidial germination and polarized growth, once conidia have been phagocytized by macrophages.

As *P. marneffei* is the only dimorphic *Penicillium* species it is important to keep in mind that the dimorphic switch is not only an identifying characteristic of the species, but one of many factors that have allowed *P. marneffei* to become such a virulent pathogen of mammals. In the context of human- fungal interactions, *P. marneffei*'s genome is of interest due to the tightly controlled manner in which this fungus grows. Once gene functions have been elucidated, they may serve as potential drug targets. Some of *P. marneffei*'s genome has already been annotated, including some genes that serve as virulence factors.

Possible Virulence Factors of *P. marneffei*

Other *Penicillium* spp. have been documented as fungal pathogens of humans (Ramirez 1982). However, based on clinical diagnostic testing, *P. marneffei* is responsible for the overwhelming majority of penicilliosis cases. *Penicillium marneffei* is only one of about 40 *Penicillia* species that affect HIV-negative individuals and one of a handful that have been documented as opportunistic pathogens of those who are HIV-positive (Cooper and Haycocks 2000). This raises the question of why *P. marneffei* accounts for such a large percentage of penicilliosis cases. The answer can possibly be

found in *P. marneffei*'s physiology. The dimorphic switch itself appears to be the primary virulence factor of *P. marneffei*, as the switch is required for yeast cell morphogenesis and, hence, for systemic pathogenesis in host organisms (Canovas and Andrianopoulos 2007; Andrianopoulos 2002; Boyce et al. 2009).

Other physiological characteristics of *P. marneffei* may also play a role in its virulence. Upon phagocytosis by macrophages conidia are subjected to environmentally unfavorable conditions inside phagosomes (Pongpom et al. 2005). These organelles are typically the site of reactive oxygen species (ROS) production, including superoxide, hydrogen peroxide, and hydroxyl radicals, termed respiratory burst, during infection. As a result, these ROS act on, and ultimately destroy, the conidia and yeast cells contained within the phagosome. While the exact defense mechanism utilized by *P. marneffei* has not been completely charted, a possible answer lies in the *cpeA* gene, which encodes a catalase-peroxidase enzyme that exhibits both catalase activity (the ability to break down hydrogen peroxide into water and molecular oxygen) and peroxidase activity (the ability to use hydrogen peroxide to oxidize other compounds). While most other microorganisms are known to possess similar enzymes, although usually as independent catalase and peroxidase enzymes, some bacteria and fungi possess the bifunctional form of the enzyme. Partial experimental characterization of the *P. marneffei* yeast phase proteome has revealed an increase in the expression of catalase-peroxidase relative to that of the mold phase. In addition, the *P. marneffei* catalase-peroxidase amino acid sequence has been shown to be 45-69% identical to that found in other fungi and bacteria. Pharmacological studies have also reported similar findings (Taramelli et al. 2001). The antimicrobial chloroquine was shown to raise the intravacuolar pH of studied

phagosomes, binding the free protons within the phagosomes and possibly inhibiting the vacuolar proton- ATPase, which maintains a proton gradient across the vacuolar membrane and perpetuates an acidic intravacuolar environment. Both of the aforementioned effects contribute to raising the intravacuolar pH. Because *P. marneffei* grows optimally at an acidic pH (5.5-6.0) the increased pH most likely inhibits yeast cells' potential for survival. The observed increase in intravacuolar pH may also inhibit pH-dependent yeast virulence factors. *Penicillium marneffei* has been documented to express acid phosphatase activity, which has also been reported in other intracellular pathogens and whose optimal pH is 5. Acid phosphatase activity is suspected to aid in the survival of intracellular pathogens by limiting the previously described respiratory burst that occurs in the phagosomes of phagocytic cells. These data suggest that catalase-peroxidase plays a major role in the survival of *P. marneffei* in the macrophage phagosome, making possible for infection to continue and potentially develop into the more severe systemic penicilliosis.

In addition to catalase-peroxidase activity, *P. marneffei* also activates the copper, zinc-superoxide dismutase enzyme encoding gene, *sodA*, upon internalization by macrophages (Thirach et al. 2008). Up-regulation of this gene during yeast cell development may also aid in *P. marneffei*'s ability to survive the harsh conditions encountered during intracellular growth.

Yeast cells are also subjected to conditions of inadequate nutrient supplies within macrophage phagosomes. In order to combat such conditions, *P. marneffei* differentially expresses genes that aid in utilizing non-preferred carbon sources (Canovas and Andrianopoulos 2006; Thirach et al. 2008). Experimental data has proven the differential

expression of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the glyoxylate cycle enzyme isocitrate lyase encoding genes, *gpdA* and *acuD*, respectively, after induction of dimorphic switching (Thirach et al. 2008). Mold phase cells exhibited significantly higher levels of GAPDH than their yeast phase counterparts. Conversely, yeast phase cells produced more isocitrate lyase than those of the mold phase. Because GAPDH has been associated with glycolysis, a pathway typically implemented under conditions that provide organisms with sufficient sources of carbon, the down-regulation of *gpdA* in yeast cells suggests that intracellular macrophage conditions are not conducive to the growth of *P. marneffei* during infection. However, in typical evolutionary fashion of many pathogens, *P. marneffei* has evolved to overcome the hostile environment of its host's phagocytic cells by utilizing alternative sources of energy. During yeast cell development *acuD* is expressed. Consequently, isocitrate lyase is produced, allowing implementation of the glyoxylate cycle. Isocitrate lyase affords *P. marneffei* the ability to bypass one of two decarboxylation steps in the TCA cycle by hydrolyzing isocitrate, a six carbon molecule, to succinate, a four carbon molecule, and glyoxylate, a two carbon molecule (Canovas and Andrianopoulos 2006; Thirach et al. 2008). *Penicillium marneffei*, as well as other intracellular pathogens, is suspected to use glyoxylate or other two carbon molecules, perhaps products of fatty-acid degradation, in the glyoxylate cycle in gluconeogenesis. Similarly, the *P. marneffei acuD* gene has also been shown to be required for growth on gluconeogenic carbon sources, including acetate and fatty-acids, and is independently regulated via dimorphic switching, partially controlled by the transcriptional activator AbaA. Hence, the expression of *acuD*

increases, along with microbial virulence, during the infectious stage of growth in *P. marneffei*.

While many more possible virulence factors are likely to exist, these genes have been experimentally shown to be up- or down-regulated depending upon the dimorphic switch, which is itself a characteristic that increases the virulence of this already pathogenic organism. Further experimentation focusing on *P. marneffei*'s genome is imperative to understanding the mechanisms and pathways involved in pathogenesis.

The Septins

In addition to the cell wall synthesizing enzymes and other constitutive, cell wall molecules is the family of highly, evolutionarily conserved family of proteins known as the septins. Septins are found in a wide range of organisms, ranging from yeast, to mice, to humans (Pan et al. 2007). The roles of septins, which include the formation of septa, polarized growth, initiating sites of new growth, and the processes of cellular division, are similar in most eukaryotes, excluding plants (Momany et al. 2001; Longtine et al. 1996). Septins were first characterized in the budding yeast *S. cerevisiae* in association with the mother- bud neck created during cellular division, and were later recognized as products of several cell-division-cycle genes (*CDC3*, *CDC10*, *CDC11*, and *CDC12*) in *S. cerevisiae* (Byers and Goetsch 1976; Haarer and Pringle 1987; Kim et al. 1991; Ford and Pringle 1991). Near the cytoplasmic surface of the mother- bud neck lies a ring comprised of filaments, each approximately 10 nm long and characteristically observed in the mother- bud neck. It was in this ring that the septins were originally documented. Interestingly, experiments on temperature sensitive *S. cerevisiae* mutants grown at the

non-permissive temperature resulted in the suppression of that particular septin gene, revealing that if any one of the four septins that form the mother- bud neck complex were not to be expressed, not only would cells not divide properly, but the septin ring also fails to form, resulting in chains of elongated cells with multiple bud sites as well as multiple nuclei (Byers and Goetsch 1976). Such evidence suggests that septins play a larger role than merely transiently interacting with the eukaryotic cytoskeleton.

As may be expected with genes involved in cellular division, *S. cerevisiae CDC* genes have since been revealed to have homologues in many other species of fungi and animals, including the filamentous fungus *Aspergillus nidulans* (Momany et al. 2001; Lindsey et al. 2010). The *A. nidulans CDC3*, *CDC10*, *CDC11*, *CDC12* homologues are *aspB*, *aspD*, *aspA*, and *aspC*, respectively. *Aspergillus nidulans* also contains a fifth septin found only in filamentous fungi, *aspE*, and its septins share 35% homology in their amino acid sequences, most of which is contained in the central regions, a property of all septins (Momany et al. 2001; Juvvadi et al. 2011). Structurally, these proteins contain three predicted P-loop domains with GTPase activity, which suggests septin complex formation and function may be dependent upon nucleotide binding and subsequent hydrolysis (Longtine et al. 1996; Saraste et al. 1990). Another secondary structure, the coiled- coil domain, is also present in most septins, excluding *aspD*, near their C-terminus and is thought to play a role in septin- septin or other septin- protein interactions (Lupas et al. 1991). Expression experiments (Momany et al. 2001) have shown that in synchronized, conidiating cultures *aspB* was expressed the greatest, followed by *aspC*, *aspA*, *aspE*, and finally *aspD*.

While a difference in expression of individual septins is evident, it must be emphasized that the septins do actually interact with one or all of the other septins, including co-localization and supplemental expression in instances when deletion strains are created. Experimentation using green fluorescent protein (GFP)- tagged AspA and AspC, as well as *aspA*, *aspC*, and *aspAaspC* deletion strains, has shown both proteins play similar roles in cellular growth and development in *A. nidulans*, where they form the characteristic ring-like structure through which newly formed cellular bodies grow, and, in the cases of *aspA*, *aspC*, and *aspAaspC* deletion strains, either provide a partial function to support their partner's absence or function improperly due to some level of dependence on their septin partner (Lindsey and Momany 2006; Lindsey et al. 2010). GFP- tagged AspA and AspC were observed localizing to sites of cell wall manipulation, including sites of germ tube emergence, newly branching hyphae, septation, at the tips of mitotically active hyphae, and even during development of new conidiophore cell layers. However, while AspA and AspC are both certainly involved in septation, they are not necessarily required for septation to be completed or even present. For instance, *aspA* deletion strains exhibited septation levels, albeit delayed, similar to the wild-type strain (Lindsey et al. 2010). In contrast, the *aspC* deletion strain exhibited only about a third of the level of wild-type septation, indicating that, while AspA and AspC do co-localize in many instances, AspC plays a more specialized role in septation than does AspA. Furthermore, deletion of either or both *aspA* or *aspC* results in both the disruption of spatial patterning of germ tube emergence and an increase in the total number of germ tubes per germinating conidium and hinders proper conidiation. Subsequently, in an effort to uncover any degree of co-localization between AspA and AspC, *A. nidulans*

aspA deletion strains with a GFP- tagged AspC and *aspC* deletion strains with a GFP-tagged AspA were created. Photographs taken via fluorescence microscopy revealed that AspC-GFP failed to localize in *aspA* deletion strains. Similarly, AspA-GFP localized abnormally in *aspC* deletion strains. Additional studies inserting *aspC* into *S.* cerevisiae *CDC12* deletion strains have shown that the Cdc12 homologue can actually alter morphology in budding yeast to resemble that of filamentous fungi (Lindsey et al. 2010). Such *S. cerevisiae* mutant strains resembled hyphal growth, with characteristically large, fuzzy colonies with a high percentage of elongated, filament-like cells, as opposed to the smaller, smoother colonies with ovoid or round shape observed in yeast growth. Further analysis revealed that the native AspA homologue, Cdc11, co-localized with the *aspC* product at the bases of the filament-like cells, termed atypical pseudohyphae.

Like *aspA* and *aspC*, the *CDC3* homologue, *aspB*, is also involved in the formation of septa, branches, and asexual reproductive structures, with subtle variations (Westfall and Momany 2001; Momany and Hamer 1997). AspB has been documented localizing to forming septa in a step-wise and regulated manner (Westfall and Momany 2001). In *A. nidulans*, a single ring of filamentous actin (F-actin) is first formed, a step proven to be crucial, as mycelia treated with F- actin depolymerizing agents were shown to lack the typical localization pattern of AspB to septation sites. Once the F- actin ring is formed, AspB co-localizes in a ring- like structure to the septation site. After the F-actin and AspB rings have co-localized, deposition of cell wall material begins, invagination of the cell wall is initiated, and the forming septum can be visualized. In budding yeast or animal cells this invagination would continue until two daughter cells were produced. However, in filamentous fungi, contraction of the actin ring does not

lead to complete cytokinesis. Cytokinesis in filamentous fungi results in septation of hyphae and compartmentalization of cellular organelles, including cytoplasm and the mitotically produced nuclei. Unlike the actin ring, though, AspB does not contract. Instead, the AspB ring separates into two distinct rings, each bordering one side of the septum. As invagination continues, the actin ring contracts until it is eventually no longer associated with the septum. Nearing the completion of septation, the basal ring of AspB disappears, and only the apical AspB ring is left, until it too is lost. Once septation has been completed, neither actin nor AspB are associated with the septum. Related analysis of AspB and chitin rings in hyphal tip cells also showed a pattern of maturation within the process of septation itself, with the most mature septa near the apical end of the cell and the least mature septa near the basal end. In addition to septation, AspB may also play roles in branching and conidiophore development; serves as a molecular marker for branching, as it localizes premitotically to new branch points; is present at sites of secondary germ tube emergence, but not of the primary germ tube, from germinating conidia; and localizes at the aerial hypha/metulae, metulae/phialide, and the phialide/conidium interfaces, but disappears as the descendant cell types mature.

The fourth, and final, known *S. cerevisiae* homologue in many filamentous fungi is *aspD*. While documentation of the AspD septin is scant, some brief experimental characterization has been conducted in the human pathogenic fungus, *A. fumigatus* (Juvvadi et al. 2011). Experimentation using GFP- tagged AspD has revealed that AspD localizes at many sites as ring-like structures or associated with discontinuous tubules in the cytoplasm of the hyphal body. GFP- tagged AspD also was present at both forming

and mature septa, as a layer in the vesicle of the aerial hypha at the point of phialide emergence, and in phialides at the point of conidiogenesis.

AspE, a septin found exclusively in filamentous fungi, has been briefly characterized in *A. fumigatus* (Juvvadi et al. 2011). GFP- tagged AspE, rather than exhibiting localization patterns seen in septins AspA, -B, or -C, appeared to localize to cytoplasmic networks of long, interconnecting filaments, which resemble microtubule networks. Another contrasting observation of GFP- tagged AspE was that characterized hyphae lacked the characteristic septin ring. However, AspE was observed at newly formed septa.

Transformational Mutagenesis

While many elements of *P. marneffei*'s biology have remained elusive, some studies have been successful in creating ways of revealing gene function in *P. marneffei*. *Agrobacterium tumefaciens*, the Gram-negative bacterium responsible for producing crown gall tumors in plants, has been put to use in transformation studies (Michielse et al. 2005). *Agrobacterium tumefaciens* contains a DNA sequence coding for tumor-inducing (Ti) factors. Part of the sequence is transferred to a host plant cell in a single stranded form as a plasmid, where it is integrated into the plant's genome. The transferred DNA sequence is termed T-DNA. T-DNA sequences contain genes encoding plant growth regulating enzymes and a virulence region, which not only houses the *vir* genes needed for tumorigenicity, but may also encode genes responsible for the construction, transport, and integration of T-DNA into the host genome. In addition, the Ti plasmid is surrounded by repeated sequences acting as signals for delivery of DNA to the host cell.

The presence of *A. tumefaciens* alone, however, is not enough for DNA transfer to occur. *vir* genes must be induced prior to co-cultivation of *A. tumefaciens* and host strains. In order to induce the *vir* genes phenolic compounds, such as acetosyringone, have been used. Although *A. tumefaciens* has been thoroughly used in transformations of other fungi, *Agrobacterium tumefaciens* – mediated transformation (AMT) has recently been applied to *P. marneffei* in several studies, yielding morphological mutants (Zhang et al. 2008). Zhang et al., (2008) transformed *P. marneffei* to create at least seven morphological mutants. Sequence analysis of those mutants revealed mutations in genes whose products included transporter proteins, transcription regulators, synthases, kinases, and revealed an unknown mutation that caused an excess of mucus to be produced at the surface of the colony. A further improved AMT system (Kummasook et al. 2010) created many mutants, a number of which remain uncharacterized. One of these mutants is the subject of the present study.

Objective

Each of the five *asp* genes in *Aspergillus* species have been shown to be differentially expressed and their respective products localized to varying locations in the filamentous fungal cell. Based on previous experiments with the related ascomycetous fungi *A. nidulans* and *A. fumigatus*, a degree of functional overlap has been revealed between septins, with some physiological processes still occurring in strains with one or another deleted septin. The current body of reported data suggests that in the AMT-generated *aspC* mutant of *P. marneffei*, not only will the level of expression of *aspC* decrease relative to the wild-type, but the remaining four septins will be up-regulated in a compensatory effort to provide proper homeostatic control for the cell.

The first of two major goals in this proposal is to phenotypically characterize the *aspC* mutant of *P. marneffei*, beginning with its most superficial macroscopic qualities, including color, degree of conidiation, and degree of colony growth, followed by a more detailed, microscopic examination, such as conidiophore structure and any potentially observable phenotypes due to the disruption mutation of the *aspC* gene. Additionally, because septins are extensively involved in processes requiring cell wall manipulation, both the wild-type and mutant strains will be cultured in an environment that stresses the cell wall using two polysaccharide-binding dyes, Congo red (CR) and Calcofluor white (CW). Chitin biosynthesis, structure, and morphology in the Basidiomycete *Mucor rouxii* was strongly inhibited or reduced by varying concentrations of the polysaccharide-binding dye Congo red (CR) *in vitro* (Bartnicki- Garcia et al. 1994). Higher concentrations of CR stopped chitin formation entirely or formed a slimy, brightly colored dye-chitin complex. Subjecting *M. rouxii* to lower concentrations of CR resulted in a red precipitate and nearly colorless solution, indicating chitin had completely bound the CR. Exposure to CR also produced much thinner chitin fibrils relative to chitin fibrils formed in the absence of CR and a gel-like substance, rather than the crystalline structure of chitin assembled in the absence of CR. Similarly, CR has also been used as a microscopic fluorescence growth indicator of hyphal growth in *Aspergillus niger* (Matsuoka et al. 1994). Subjecting hyphae to increasing concentrations of CR yielded morphological anomalies including swelling and curling of hyphae. Fluorescence measurements also indicated that as long as hyphae were growing (i.e., cell wall surface area and the number of chitin molecules increased, thus increasing the number of CR binding sites) the fluorescence of CR-bound hyphae increased linearly with time until

miconazole, a hyphal growth inhibitor, was added. Examined sections of hyphal cell walls displayed greater increased fluorescence levels behind the apex than in the apex cell walls, suggesting an uncertain binding affinity of CR for chitin and providing some evidence as to the type and location of chitin bound by CR. However, the CR-chitin binding event occurs in a stepwise manner (Herth 1980). Because the crystallization process is disturbed, it appears that CR has a greater binding affinity for polymerized chitin than for the larger, and more elaborate, crystalline structure of side-linked chitin microfibrils. Thus, CR-chitin reactions usually follow this binding pattern. Calcofluor white (CW), another polysaccharide- binding dye, has also been shown to have similar effects on both filamentous fungi and budding yeasts (Roncero and Duran 1985). Subjecting *Geotrichum lactis* to CW resulted in cellular lysis at mycelial tips, while *Saccharomyces cerevisiae* exposed to CW resulted in incomplete cytokinesis. Subjecting both the wild-type and mutant strains to CR and CW should lead to an increase in septin expression.

The second goal of this proposal is to document the differential expression, if any, of each *asp* gene between the mold and yeast phases of both the wild-type and mutant strains cultured under normal conditions (e.g., cultured in Sabouraud dextrose agar [BD Biosciences] ((SAB); 20g/L dextrose and 10g/L peptone) or minimal medium ((MM); 20mL 50x salt solution; 1mL trace elements (40mg $Na_2B_4O_7 \cdot 7H2O$; 400mg $CuSO_4 \cdot 5H2O$; 800mg $FeSO_4 \cdot 7H_2O$; 800mg $NaMoO \cdot 2H_2O$; 8g $ZnSO_4 \cdot 7H_2O$); 10g glucose; and 66.3mL of 0.3M L-glutamine; at a pH of 6.5) without any supplements) and under conditions which stress the fungal cell wall. Again, this may potentially reveal the roles some genes play in growth and cellular development, as well as phase transition.

The identification of the *aspC* gene's exact function brings with it more crucial evidence needed to view the larger scope of how *P. marneffei* interacts with its environment, particularly in its role as an often aggressive, opportunistic pathogen of immunocompromised individuals.

Chapter II

Materials and Methods

Strains, Maintenance, and Growth Conditions

The *P. marneffei* wild-type strain (strain F4; CBS 119456) was procured from the hemoculture of an AIDS patient from the central laboratory, Maharaj Nakorn Chiang Mai Hospital in January of 1999 (Kummasook 2010).

The *P. marneffei* strains were cultured on potato dextrose agar (PDA [Difco brand] Becton- Dickinson, Franklin Lakes, NJ) for seven to ten days at 25˚C. Conidial suspensions for further experimentation were prepared by scraping the surface of the fungal culture with a cotton swab pre-moistened with sterilized water and suspending the mycelial tissue in sterilized water. The tissue suspension was filtered through sterilized glass wool (Corning, Acton, MA) in a screen cap (BioRad Laboratories, Inc., Hercules, CA) attached to a 50 mL conical tube to yield a suspension of isolated conidia via centrifugation (Gifford and Cooper 2009). The concentration of the resultant conidial suspension was measured by counting conidia with a haemocytometer (http://www.ruf.rice.edu/~biolabs/methods/microscopy/cellcounting.html) under a phase contrast microscope. After the concentration of the conidial suspension was obtained, it was used to make dilutions as necessary. *Penicillium marneffei* stock cultures were suspended in 15- 30% glycerol and stored at -80˚C.

Penicillium marneffei mutant strain I209 was derived using a modified AMT system (Kummasook 2010; Kummasook et al. 2010). Strain I209 was later revealed to be an *aspC* disruption mutant (Eric Price, personal communication).

Morphological Analysis and Characterization

Morphological characterization of *P. marneffei* and mutant strain I209 was conducted on synchronous cultures. Both wild-type and mutant strains were inoculated onto PDA plates by collecting mycelial tissue from strains cultured on PDA and subsequently streaking that tissue with a sterilized, metal inoculating loop, then incubated for seven to ten days at 25˚C and 37˚C in order to obtain isolate colonies for the purposes of single colony observation and photographic documentation. To observe asexual developmental structures, slide cultures (Harris 1986) were prepared and photographed using differential interference contrast (DIC) microscopy.

Cell Wall Disruption Assays

For cell wall disruption experiments in liquid cultures, both the wild-type and mutant strain were subjected to each condition (e.g., incubation times and temps; chemical supplements; and control cultures with no supplements). Fifty milliliters each of SAB and MM were inoculated with conidia from both fungal strains to a final concentration of at least 1.0×10^6 cells/mL and incubated at 25˚C and 37˚C for two days. Those cultures to be supplemented with H_2O_2 (Fisher) were treated with H_2O_2 four hours before ending the incubation period (44 h) to a final concentration of 2.0 mM. Congo red (CR) (Amresco) and Calcofluor white (CW) (Sigma- Aldrich) cell wall disruption experiments were performed under the same parameters as those implemented in the H_2O_2 experiments with the exception of the final concentrations of the respective supplement. CR was added to cultures to a final concentration of 20 µg/mL. CW was added to cultures to a final concentration of 80 µM.

Cell wall disruption experiments performed on PDA plates were conducted on both strains, and the following supplements and concentrations were used: Congo red (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μg/mL); Calcofluor white (10, 20, 30, 40, 50, 60, and 70 μM); and hydrogen peroxide (0.75, 0.5, and 0.25 mM). Conidial dilutions (10 μL of 1 x 10^4 cells/mL) were pipetted onto the agar plate.

Genomic DNA Extraction

Genomic DNA was extracted according to the protocol from the ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research Corp., www.zymoresearch.com). Extracted DNA was further concentrated via ethanol precipitation (Gallagher 2010). The resulting DNA samples were subjected to spectrophotometry to measure their concentrations as double-stranded nucleotides. A dilution factor of 1:10 was used, and corresponding data was recorded.

Gel Electrophoresis

In order to assess the quality of genomic DNA, samples were subjected to agarose gel electrophoresis. To make a 1.0% agarose gel, 1.0 g agarose I (Amresco) was dissolved in a flask containing 100 mL 1 x TAE buffer (50 x TAE buffer: 242 g Tris base; 57.1 mL glacial acetic acid; 100 mL 0.5 M EDTA); to prepare 1 x TAE buffer, 20 mL of 50 x TAE buffer were added to 1 L of ddH_2O) by boiling in a microwave. While waiting for the agarose solution to cool enough to handle the flask with bare hands, the electrophoresis rigging was assembled by placing the gel tray in the electrophoresis chamber and inserting the wedges into their respective slots to ensure the agarose solution did not leak out of the gel chamber. Once the flask had cooled enough to touch,

the agarose solution was poured in to the gel chamber, the comb was inserted into the gel to create wells for loading samples, and the gel was allowed to solidify. Once solidified, the wedges were gently removed, enough TAE buffer to cover the gel by three or four millimeters was poured into the chamber, and the comb was gently removed, as to not tear or otherwise damage the solidified gel. Five microliters of each DNA sample were then individually mixed with one microliter of E-Z Vision loading dye (BioRad) on parafilm via pipetting. After the DNA and dye were mixed, the sample was loaded into its respective well. In order to ascertain the relative size of each DNA sample, either a 100 base pair (bp) or 1kbp molecular ladder was loaded into a separate well. The electrodes were then oriented appropriately so that the power supply was connected to the electrophoresis chamber. The power supply was turned on, and the samples were subjected to 120 V for about one hour or as long as it took for the tracking dye to reach about three-quarters of the length of the gel, allowing adequate separation of ladder bands. The power supply was turned off, electrodes detached, and the gel tray was gently removed from the chamber, briefly allowing any excess buffer to drain. Because E-Z Vision, which is visible under UV light, was loaded with each sample, the use of the known human carcinogen ethidium bromide was avoided prior to imaging the DNA-containing gel.

Total RNA Extraction and Purification

Wild-type and mutant strains were cultured for two days in SAB broth and liquid MM under previously described conditions. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA extraction protocol for plants and fungi was followed with the following exceptions: Steps 2 and 3 in the manufacturer's

protocol were not used. At least two RNA samples were purified from each culture, and two cDNA samples were later synthesized from each RNA sample, yielding a total of at least four cDNA samples. DNase treatment was performed using DNase I (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of each RNA sample was measured via spectrophotometry and the corresponding data was recorded. The procedure for measuring RNA sample concentrations was the same as for genomic DNA, with the exception of the dilution factor, which was 1:100 for RNA samples.

cDNA Synthesis, and Qualtiy Control Experiments

Purified total RNA was used as a template for cDNA synthesis using the protocol for oligo$(dT)_{20}$ primers in the iScript Select cDNA Synthesis Kit (BioRad). An additional cDNA synthesis was performed in parallel using the protocol for cDNA synthesis with anchored oligo$(dT)_{18}$ primers in the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) in order to compare the efficacy in cDNA synthesis of each kit. In both cases, cDNA synthesis was performed according to the manufacturer's instructions. Concentrations of cDNA samples were then measured via spectrophotometry. For the measurements of cDNA sample concentrations, the procedure was the same as genomic DNA with two exceptions: a 1:100 dilution factor, rather than a 1:10 dilution factor, was used in the measurement of cDNA sample concentrations; and cDNA samples were measured as double-stranded nucleotides. After cDNA sample concentrations were determined, cDNA dilutions were prepared to a final concentration of 50 ng/µL.

As a control experiment to determine whether or not cDNA samples were contaminated, cDNA samples (50 ng/μL), as well as genomic DNA (50 ng/μL), were subjected to a polymerase chain reaction (PCR) employing primers to amplify a portion of the calmodulin gene. To amplify the calmodulin genes, cDNA and DNA samples were subjected the following reaction: 2 μL cDNA or DNA template (50 ng/μL); 4 μL nuclease- free water; 2 μL calmodulin forward primer (10 μM); 2 μL calmodulin reverse primer (10 μM); 10 μL Go- Taq Green Master Mix, 2x (Promega, Madison, WI, USA) in a final volume of 20 μL. PCR amplification conditions were 5 min for denaturation at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at an annealing temperature of 62°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C. Because the genomic DNA calmodulin product is larger than its cDNA counterpart, the DNA PCR product would migrate through the gel more slowly than the cDNA PCR product. Any evident DNA contamination in cDNA samples ran in the 2% agarose gel resulted in the exclusion of those DNA-contaminated cDNA samples.

Two- Step quantitative (q) PCR

Once cDNA samples had been verified as non-contaminated via calmodulin control PCR, multiple cDNA samples from cultures grown under the same conditions (e.g., all wild-type mold cDNA samples from SAB cultures treated with Congo red; all mutant yeast cDNA samples from MM cultures treated with Calcofluor white; etc.) were combined with one another to yield a master stock of cDNA from both phases of each fungal strain under the various conditions to which they were subjected. A 1:100 dilution of each sample was prepared for use in the qPCR.

All samples were subjected to a qPCR of each individual septin gene, along with calmodulin gene as the control, using the same parameters. Each qPCR reaction included 2.5 µL cDNA template and 18 µL master mix, which contained 3.5 µL nuclease-free water, 2 µL each of forward and reverse primers at 10 µM each, and 10 µL iQ SYBR Green Supermix (BioRad). qPCR amplification conditions were 3 min for denaturation at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at an annealing temperature of 60°C, and 91 cycles of 1 min for extension at 50°C.

Table 1- Potentially differentially expressed genes and their corresponding primers

Gene Subjected to qPCR	Primers' Sequences
calmodulin	F 5'- TCA CAA CCT CCC TTC GAT TC -3' R 5'- TCT CTT CCT CGG AGT CGG TA -3'
aspB	F 5'- TCC GCA AAG GAT TCA ACT TC -3' R 5'- GGT CTC CAT GAG TCG TCG TT -3'
aspC	F 5'- GAC AAC CCC AAG TTC AAG GA -3' R 5'- ACG AAA ATG TAC ACC GCA CA -3'
aspD	F 5'- CGG TGA CCA AGT CAA CAA TG -3' R 5'- GTC GCT CTT CCA AGG TCA AG -3'
aspE	F 5'- GCA AGG ACC CTA CCC CTT AC -3' R 5'- CCT CGA ATT TGC TCT CCA AG -3'

Chapter III

Results

Macroscopic Observations

Observational analysis of isolated colonies and slide cultures revealed stark macroscopic contrasts between *P. marneffei* wild-type and the *aspC* mutant strains. Isolated colonies obtained from strains cultured on PDA (Figure 1) showed differences in the gross amount of conidiation, patterns of conidiation, and pigmentation of conidia. Wild-type *P. marneffei* cultures exhibited thicker mycelial tissue growth and conidia appeared to germinate faster than the I209 strain. Conidiation also revealed a difference in pigmentation between wild-type and mutant strains, with wild-type conidia presenting a drab, olive- green pigment and the I209 strain displaying a vibrant, yellow pigment. Incidentally, conidiation patterns were also noticeably different from one another. While isolated wild-type colonies conidiated in a seemingly uniform pattern, isolated colonies of the I209 strain appeared to conidiate distally at first and progressively conidiate in a proximal, concentric pattern. In stark contrast to *aspC* mutant cultures incubated at 25°C, no growth was observed on PDA plates incubated at 37°C. However, wild-type *P. marneffei* growth appeared normal (no photographs were taken as evidence). In addition to unsupplemented PDA plates, experiments using PDA plates supplemented with H_2O_2, CR, and CW exhibited no growth of the *aspC* mutant. Thus, further experimentation on PDA and alternative solid media at 37°C were abandoned.

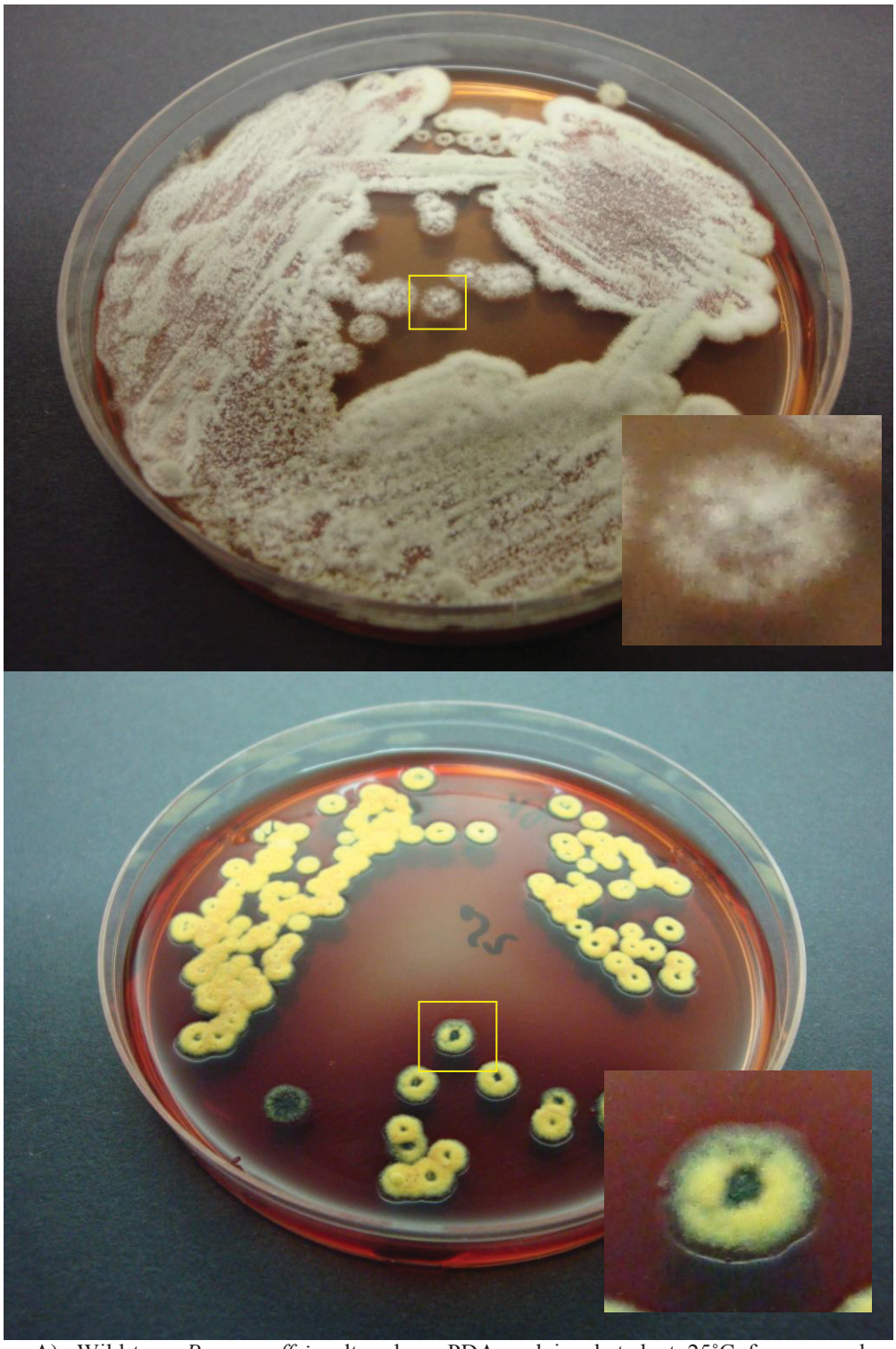

Figure 1. A), Wild-type *P. marneffei* cultured on PDA and incubated at 25˚C for seven days; B), *Penicillium marneffei* mutant I209 cultured on PDA and incubated at 25˚C for seven days; A and B), in the corner of each photo is a close- up of an isolated colony of the respective strain.

Sensitivity Plate Assays

Cell wall disruption assays were monitored from the third day of incubation to the seventh, and the radial growth was measured in millimeters. All plate assays included a negative control, that being PDA with no supplement. Initially, a lag in growth was absent at 10, 9, 8, 7, 6, and 5 µg/mL of CR and also at 0.75 mM H_2O_2 (Figures 2-6). This was confirmed by the increase in radial growth recorded as the concentrations of each supplement decreased, relative to the PDA- only negative control. However, the CW plate assay did not provide the expected results (Figures 7 and 8). Rather than the radial growth of each colony being slowed at higher CW concentrations and gradually increasing as the CW concentration decreased, as was seen in the H_2O_2 and CR plate assays, radial growth of both the wild-type and *aspC* mutant grew steadily, without any obvious or evident CW- related hindrance relative to the negative control.

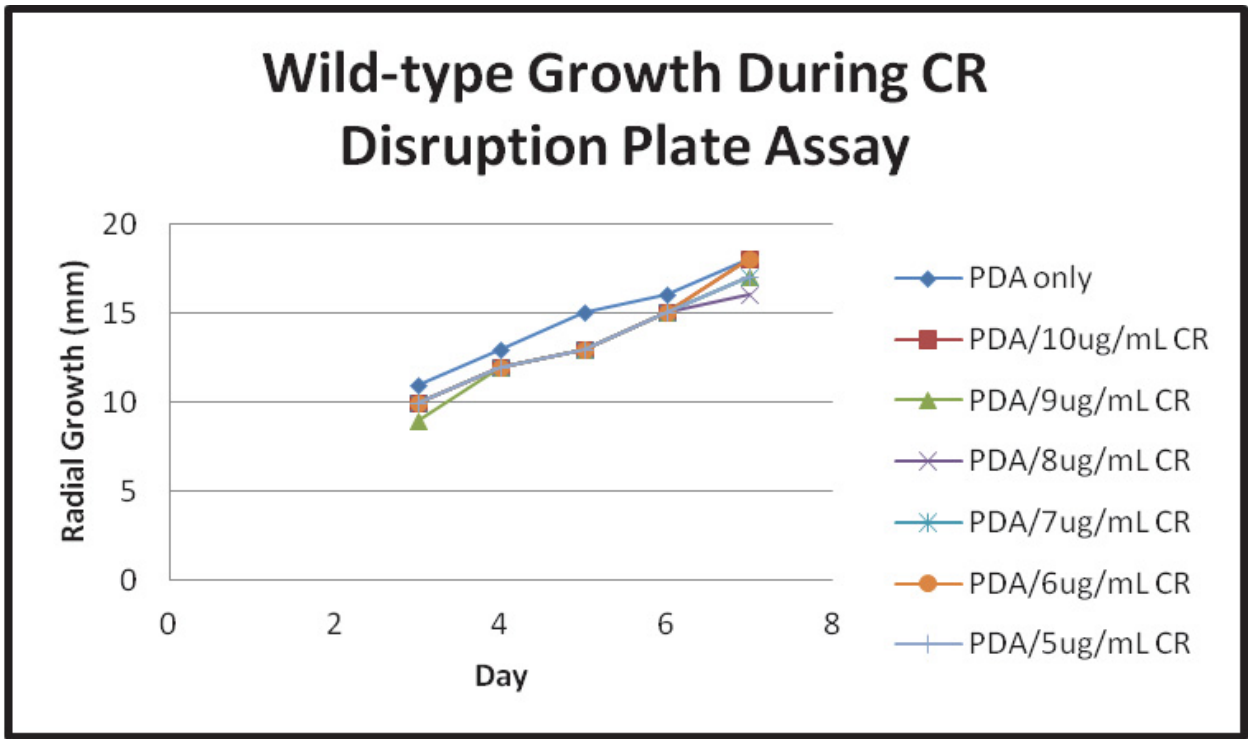

Figure 2. Radial growth of the wild-type strain over a 5 day period during CR disruption plate assay.

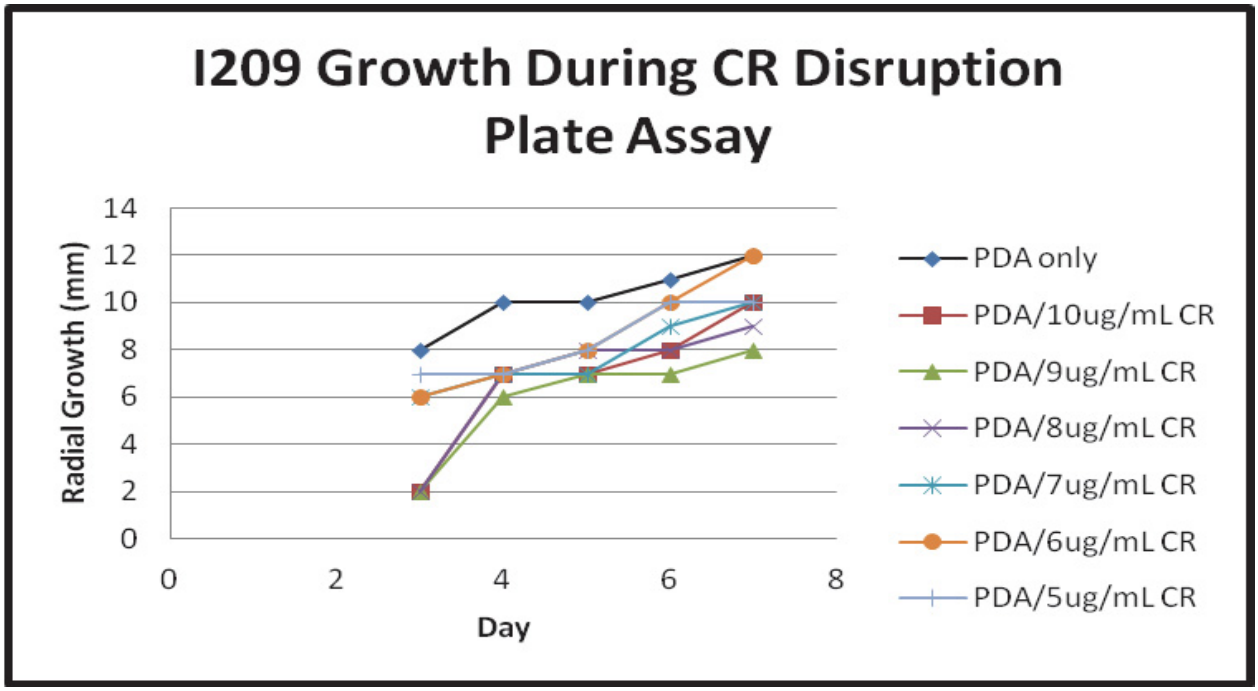

Figure 3. Radial growth of the *aspC* mutant over a 5 day period during CR plate disruption assay.

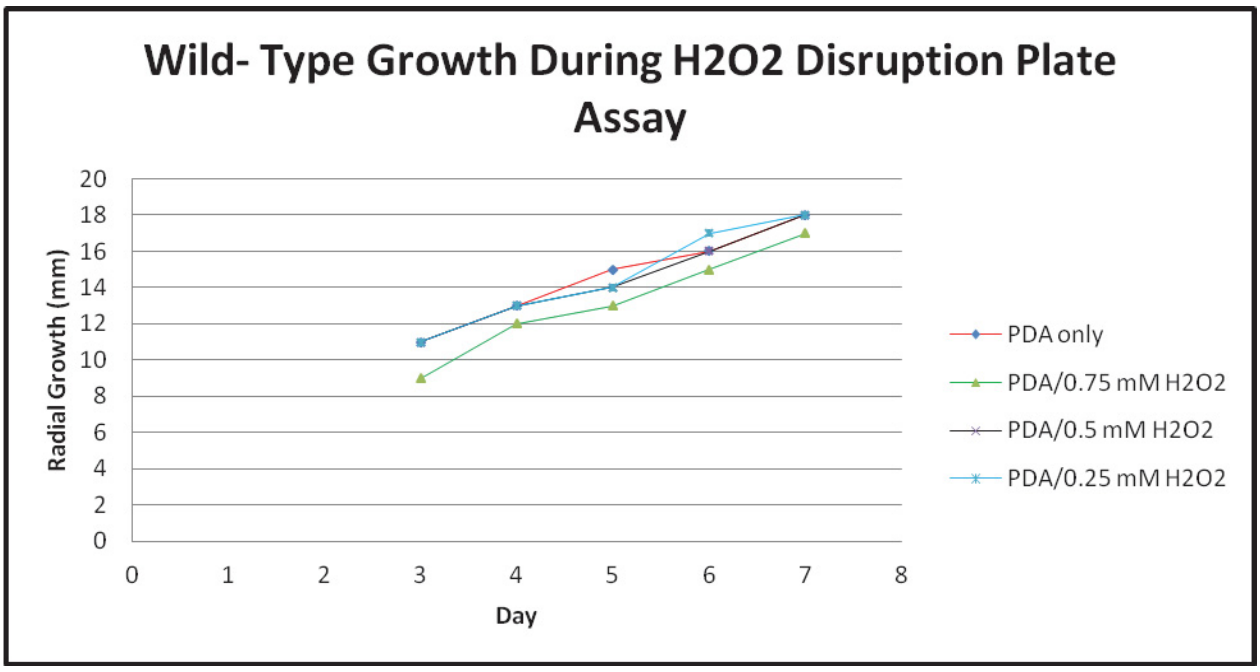

Figure 4. Radial growth of wild-type over a 5 day period during H_2O_2 disruption plate assay.

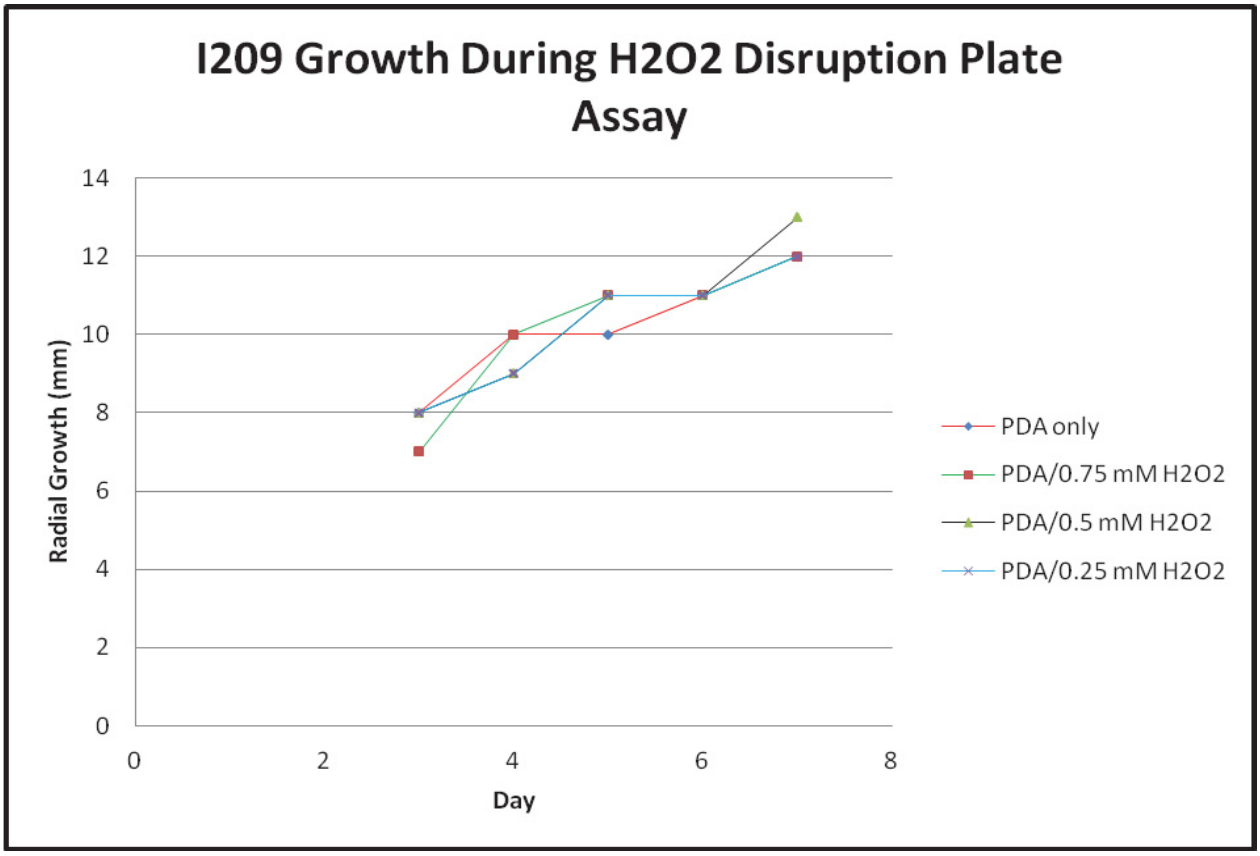

Figure 5. Radial growth of *aspC* mutant over a 5 day period during H_2O_2 disruption plate assay.

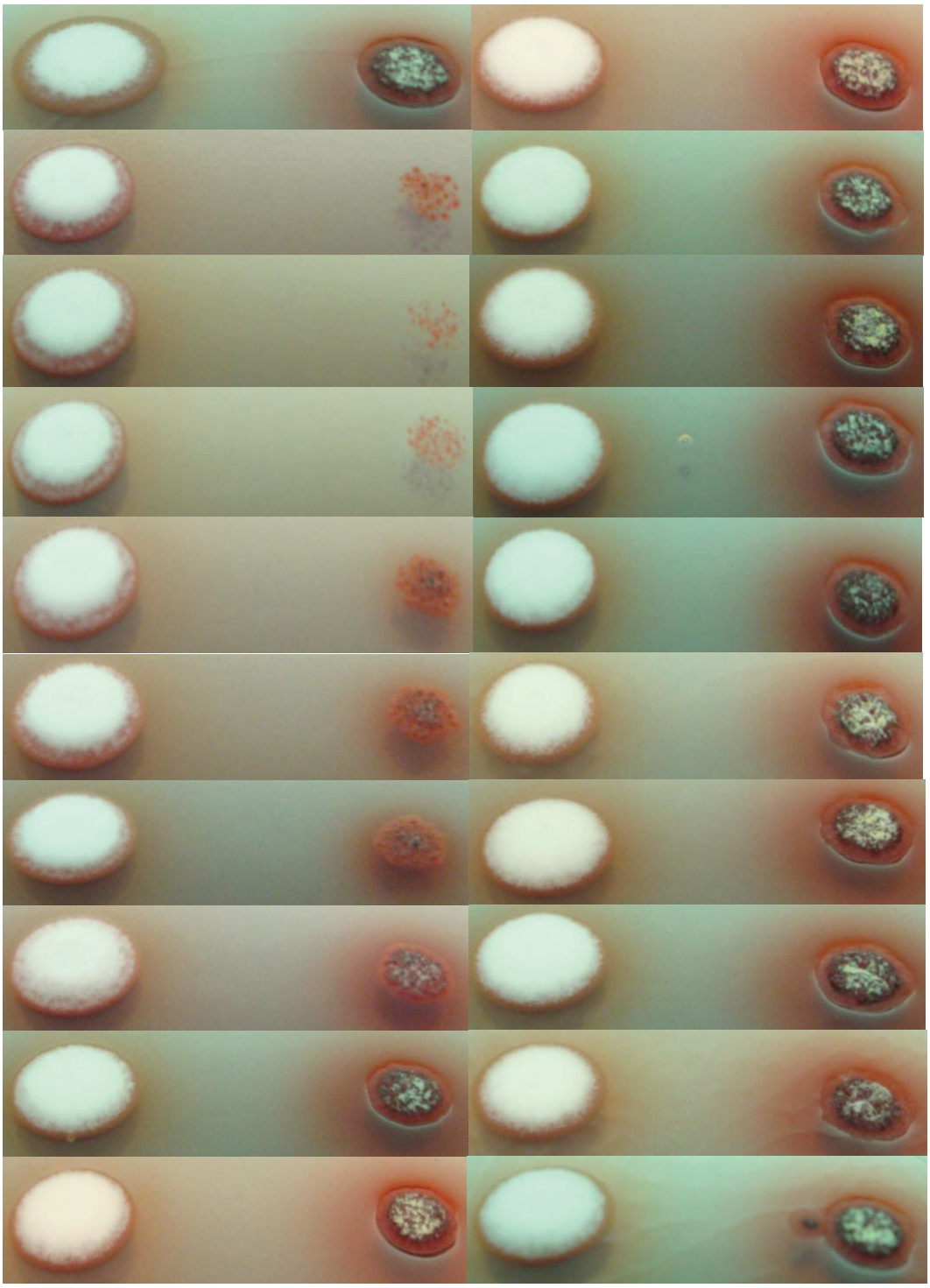

Figure 6. Cell wall disruption plate assay using CR supplemented PDA. Wild-type (left) and I209 (right) after incubating at 25°C for 4 days. The left column begins at the top with the negative control (PDA only) plate continuing with 10, 9, 8, 7, 6, 5, 4, 3, 2 µg/mL. The right column begins at the top with 1 µg/mL continuing with 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 µg/mL.

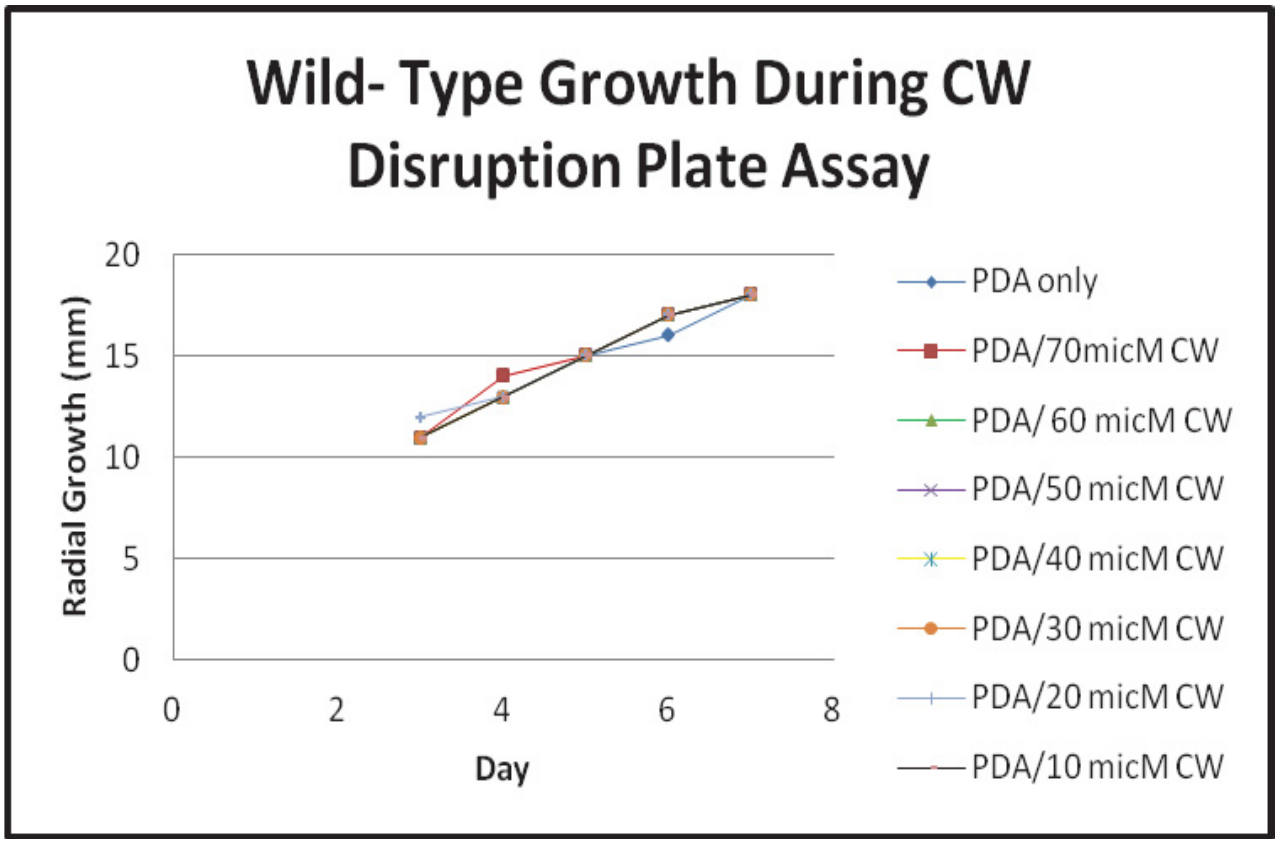

Figure 7. Radial growth of wild-type over a 5 day period during CW disruption plate assay.

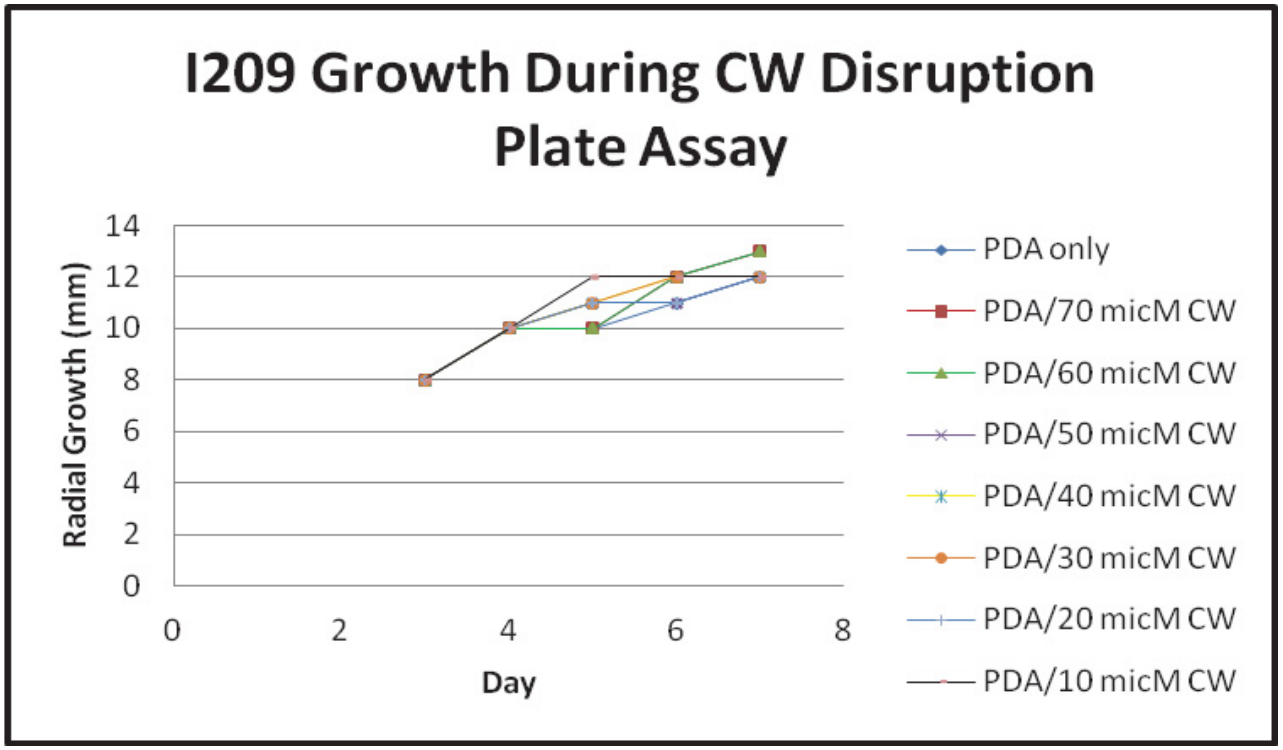

Figure 8. Radial growth of *aspC* mutant over a 5 day period during CW disruption plate assay.

Microscopic Observations

The most revealing morphological differences were observed via imaging mounted slide cultures of wild-type and *aspC* strains (Figures 9 and 10, respectively). Wild-type *P. marneffei* exhibited growth and development already well- characterized in primary and secondary literature. Hyphal cells developed into foot cells, which continued to grow apically until the asexual developmental program was triggered, consequently leading to conidiation (Canovas and Andrianopoulos 2007). Extensive conidiation was routinely observed in the wild-type, both as loose, single and long chains and those still attached to the conidiophore itself. Several forms of conidiophores were observed in the wild-type cultured on PDA, with some conidiophores producing several phialides, each with a chain of conidia, while others developed only one or two phialides from which a chain of conidia budded.

Few of these characteristics were observed in the *aspC* mutant. The *aspC* mutant displayed several traits divergent from those of the wild-type and unique to its mutation. Primarily, conidiophores were markedly different. *aspC* mutant conidiophores did not characteristically exhibit the developmentally organized conidiophores seen in the wild-type. Rather, *aspC* mutant asexual development appeared to be disorganized, with a spatial arrangement highly varied from the wild-type. Nor was there an abundance of conidia, as free, single conidia or chains, attached or otherwise observed in *aspC* mutant slide cultures. In contrast to the wild-type, mutant conidiophores produce phialides that eventually come to bear a single conidium. All observed *aspC* mutant conidiophores displayed this pattern of conidiation. Observational data of the *aspC* disruption mutant

are in parallel with those data of an *aspC* deletion strain previously characterized (Lindsey 2010).

Slide culture microanalysis of wild-type cultures revealed very little difference between the negative control (PDA only) culture and those supplemented with H_2O_2 or CW (Figures 11 and 12, respectively). However, CR wild-type cultures did show some signs of decreased asexual development (Figure 13). Wild-type cultures subjected to 25 µg/mL of CR exhibited a high degree of aberrant conidiophore types compared to the negative control and also to the lower end of CR concentrations (15 µg/mL). Wild-type cultures subjected to 20 µg/mL of CR also showed some inhibition of conidiation and aberrant conidiophore types.

Similarly, the *aspC* mutant's asexual development did not seem to be affected by either CR or CW compared to the mutant control culture (Figures 14 and 15, respectively). Although, the *aspC* mutant did appear to be affected at the upper range of the H_2O_2 assay (3 mM H_2O_2; Figure 16). At 3 mM H_2O_2, mutant conidiophores generally displayed structures finer than those observed at lower concentrations.

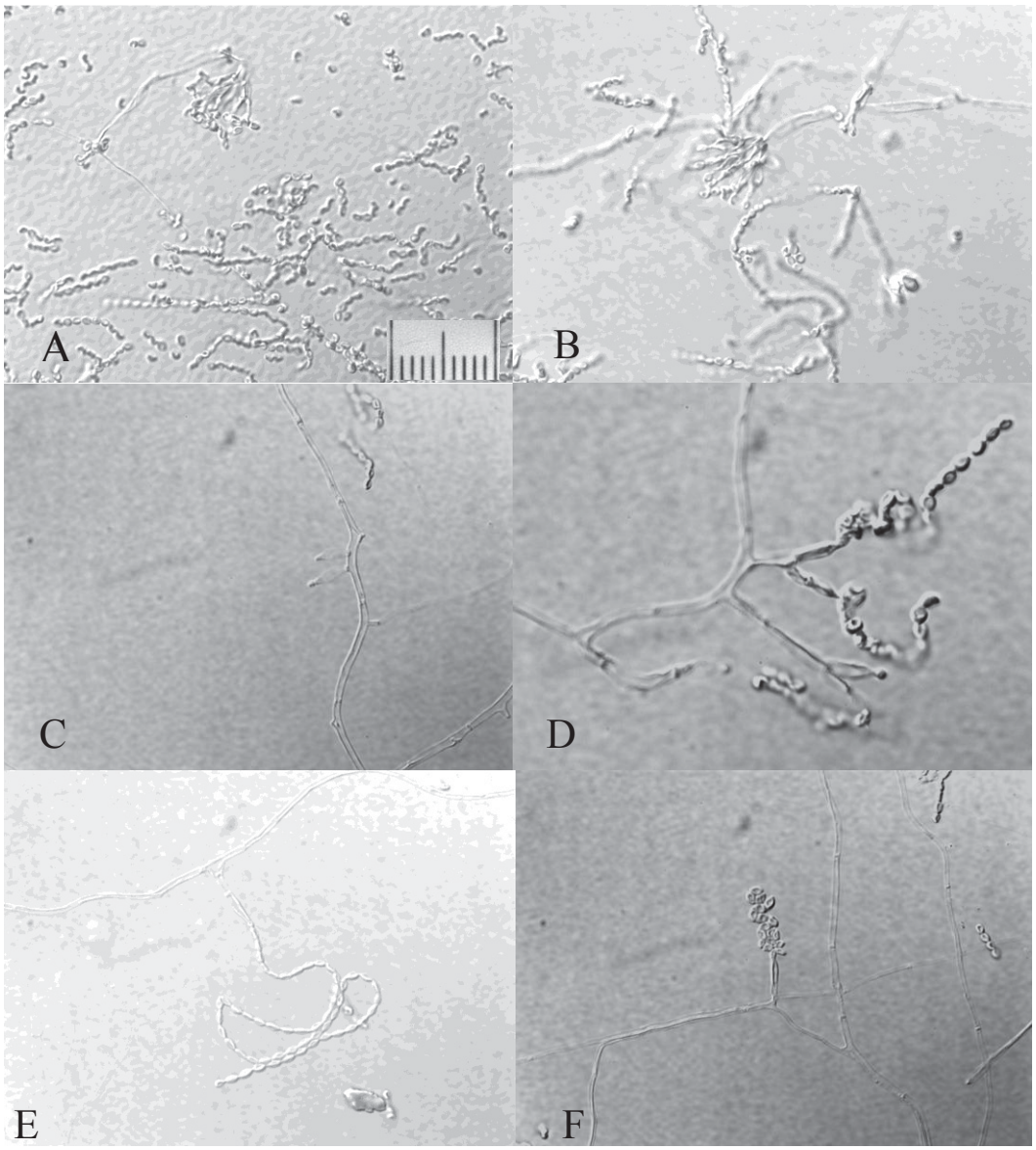

Figure 9. (A- F), 100x magnification and scale bar represents 0.1 mm; A and B), *P. marneffei* wild-type asexual developmental structures with conidiopores and dissociated conidia; C), new branching from hyphae with visible septa; D- F), aberrant conidiation observed in *P. marneffei* wild-type strain.

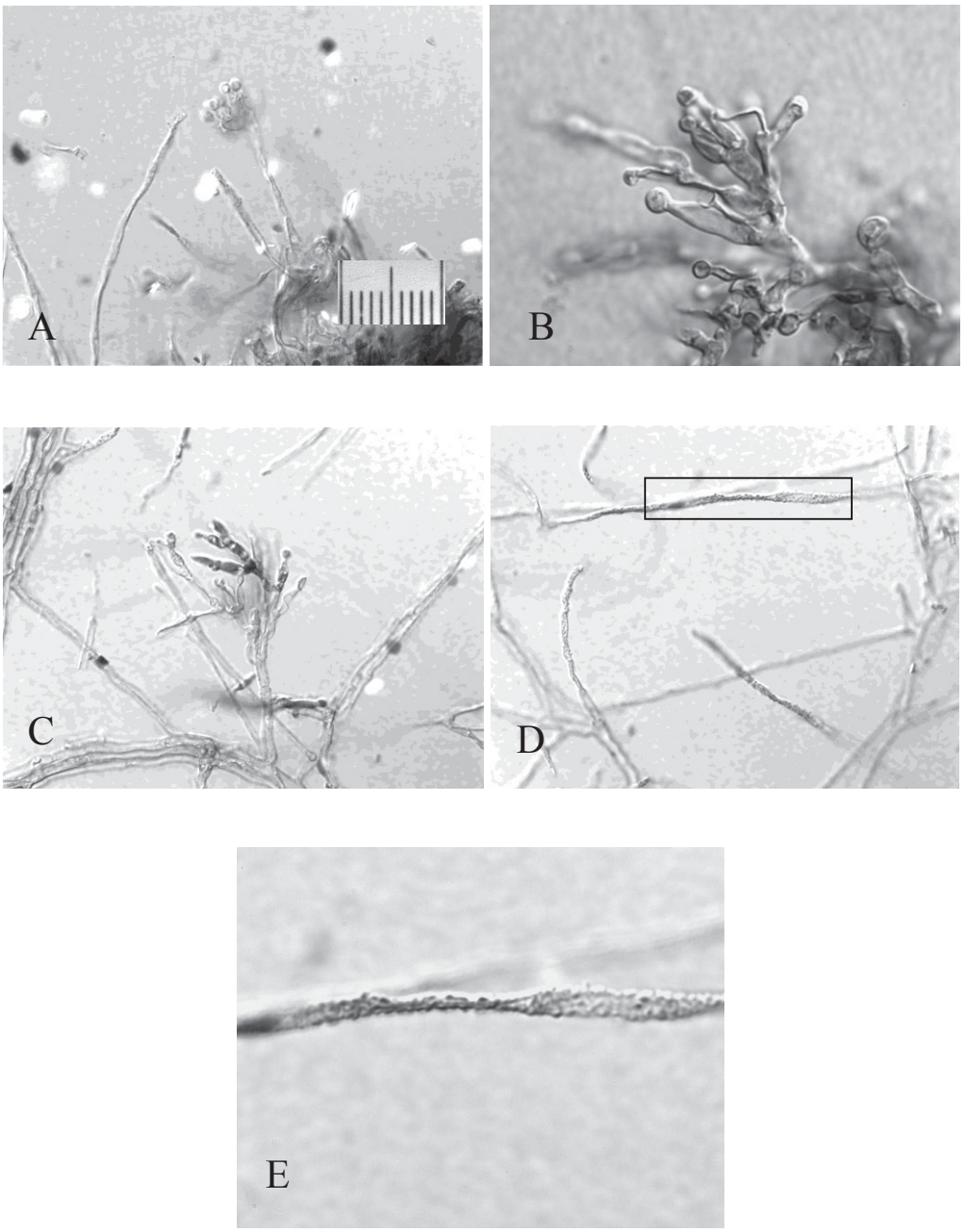

Figure 10. A- B), 100x magnification and scale bar represents 0.1 mm; A- C), I209 conidiophores; D), hyphae of I209; E), close- up of hypha from boxed area in Figure 8. D showing unidentified spots, possibly an effect of the dye- cell wall binding.

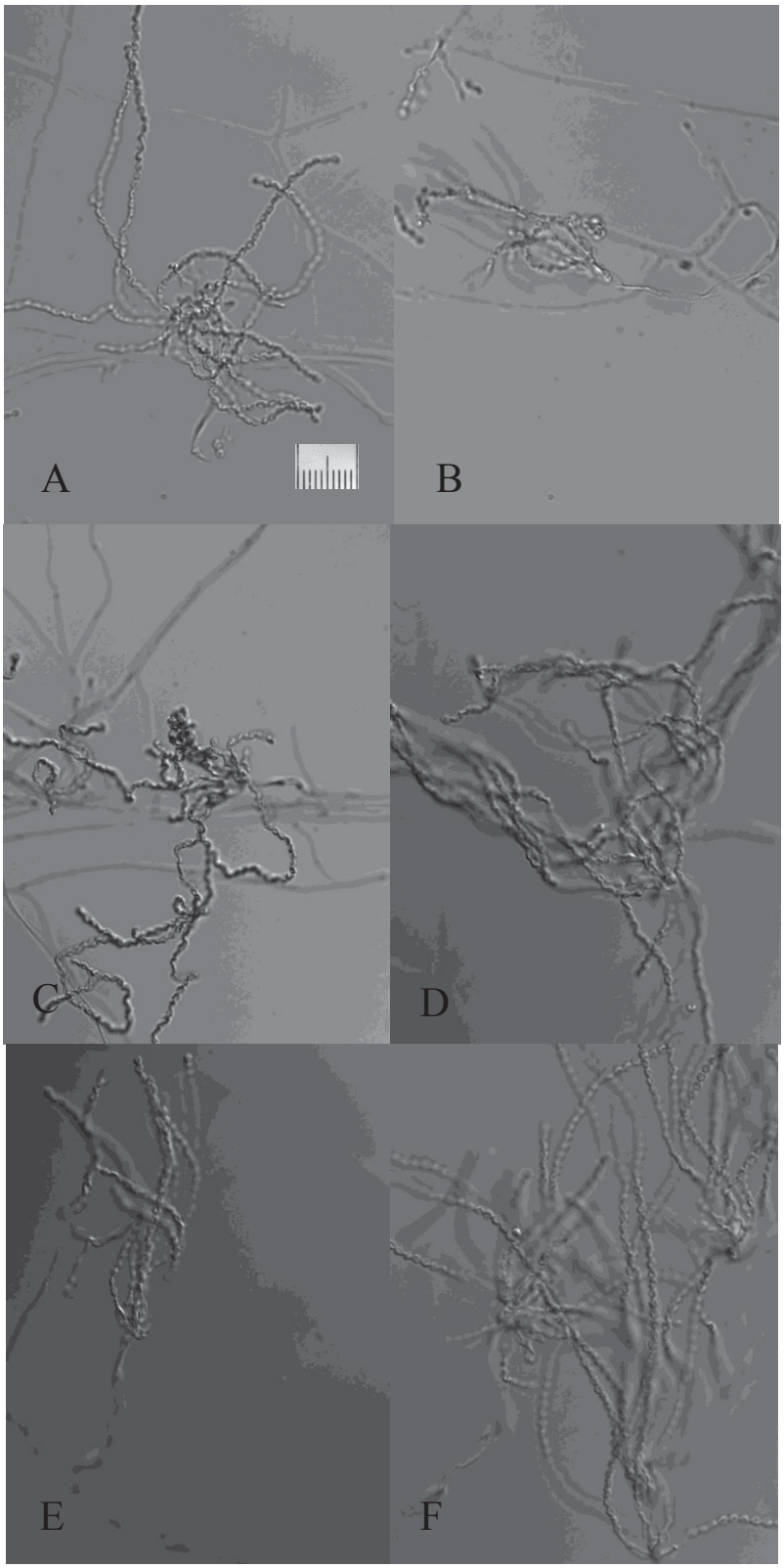

Figure 11. A- F), 100x magnification and scale bar represents 0.1 mm; *P. marneffei* wild-type H_2O_2 slide culture assay; A) PDA only; B) 1 mM H_2O_2/ PDA; C) 1.5 mM H_2O_2/ PDA; D) 2 mM H_2O_2/ PDA; E) 2.5 mM H_2O_2/ PDA; F) 3 mM H_2O_2/ PDA.

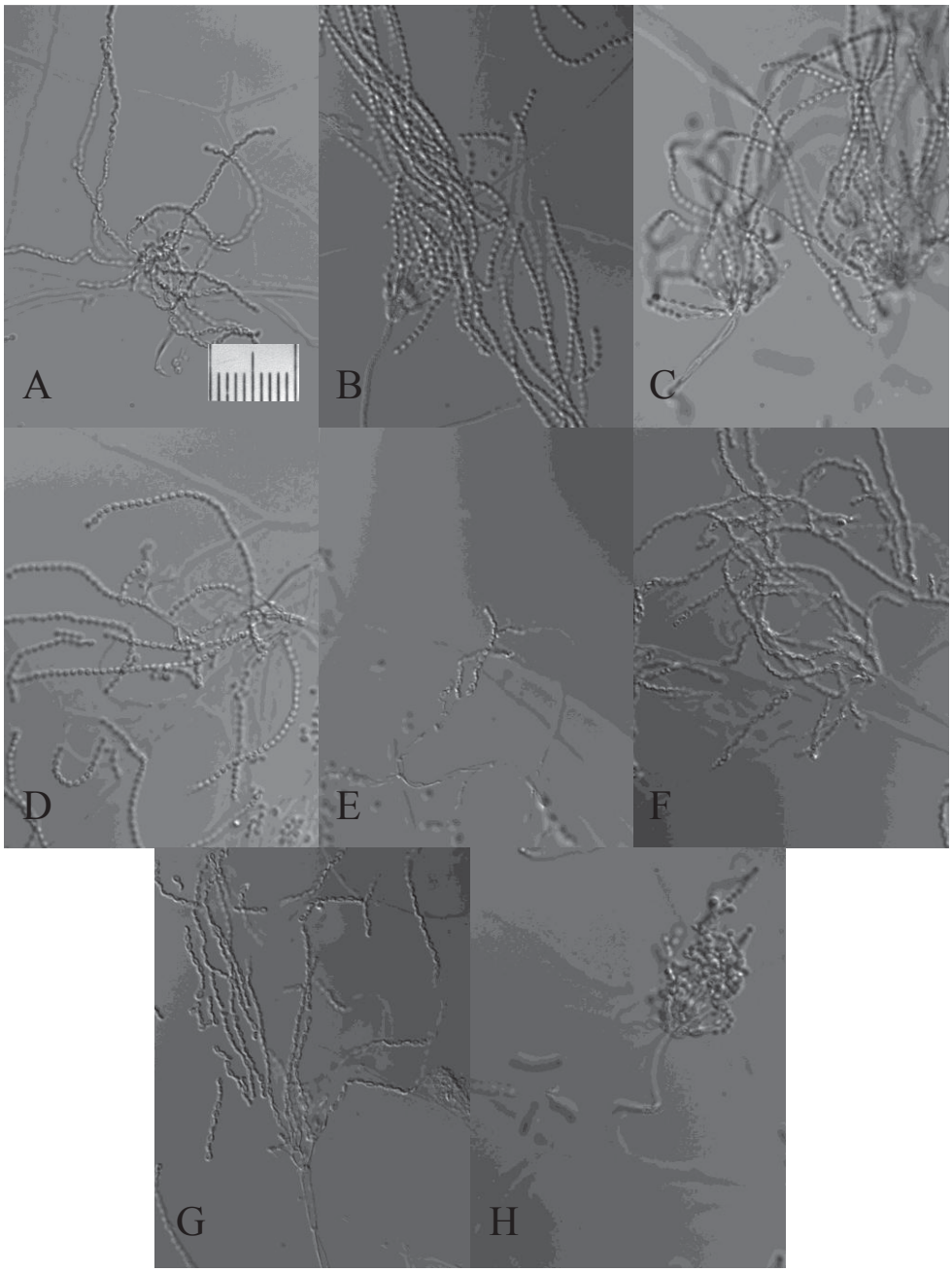

Figure 12. A- H) 100x magnification and scale bar represents 0.1 mm; *P. marneffei* wild-type CW slide culture assay; A) PDA only; B) 10 μM CW/ PDA; C) 20 μM CW/ PDA; D) 30 μM CW/ PDA; E) 40 μM CW/ PDA; F) 50 μM CW/ PDA; G) 60 μM CW/ PDA; H) 70 μM CW/ PDA.

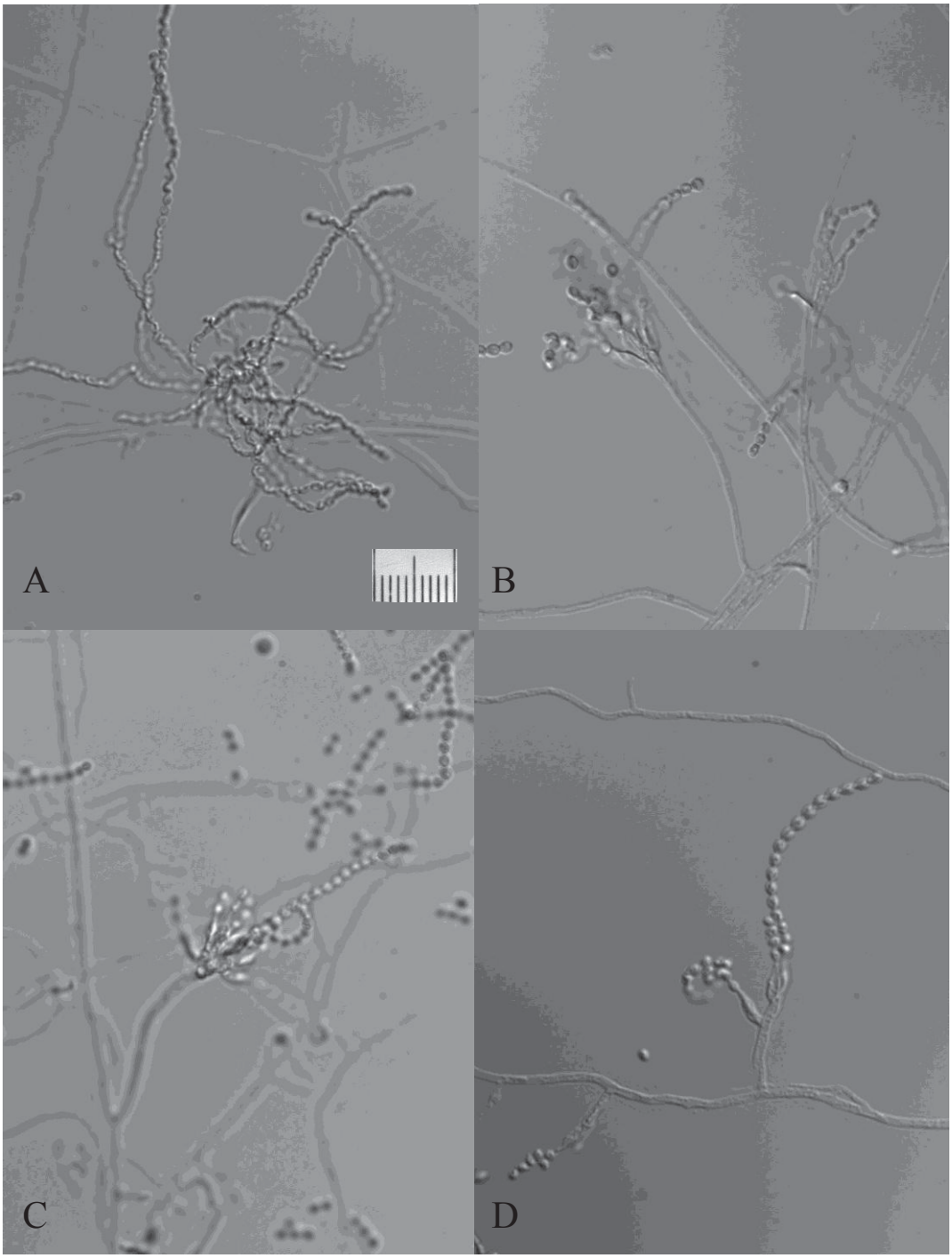

Figure 13. A- D) 100x magnification and scale bar represents 0.1 mm; *P. marneffei* wild-type CR slide culture assay; A) PDA only; B) 15 µg/mL CR/ PDA; C) 20 µg/mL CR/ PDA; D) 25 µg/mL CR/ PDA.

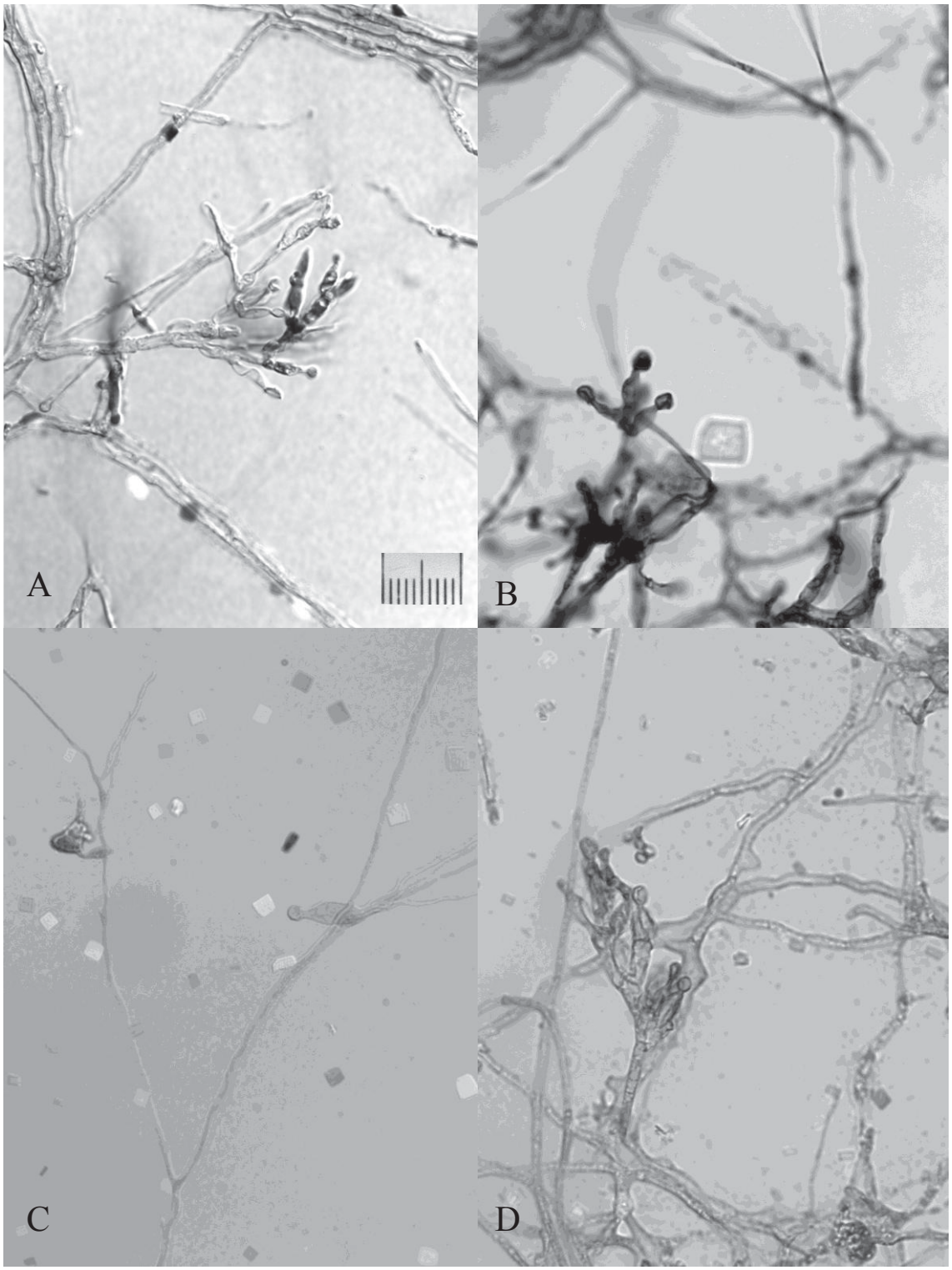

Figure 14. A- D) 100x magnification and scale bar represents 0.1 mm; *P. marneffei aspC* mutant CR slide culture assay; A) PDA only; B) 15 µg/mL CR/ PDA; C) 20 µg/mL CR/ PDA; D) 25 µg/mL CR/ PDA.

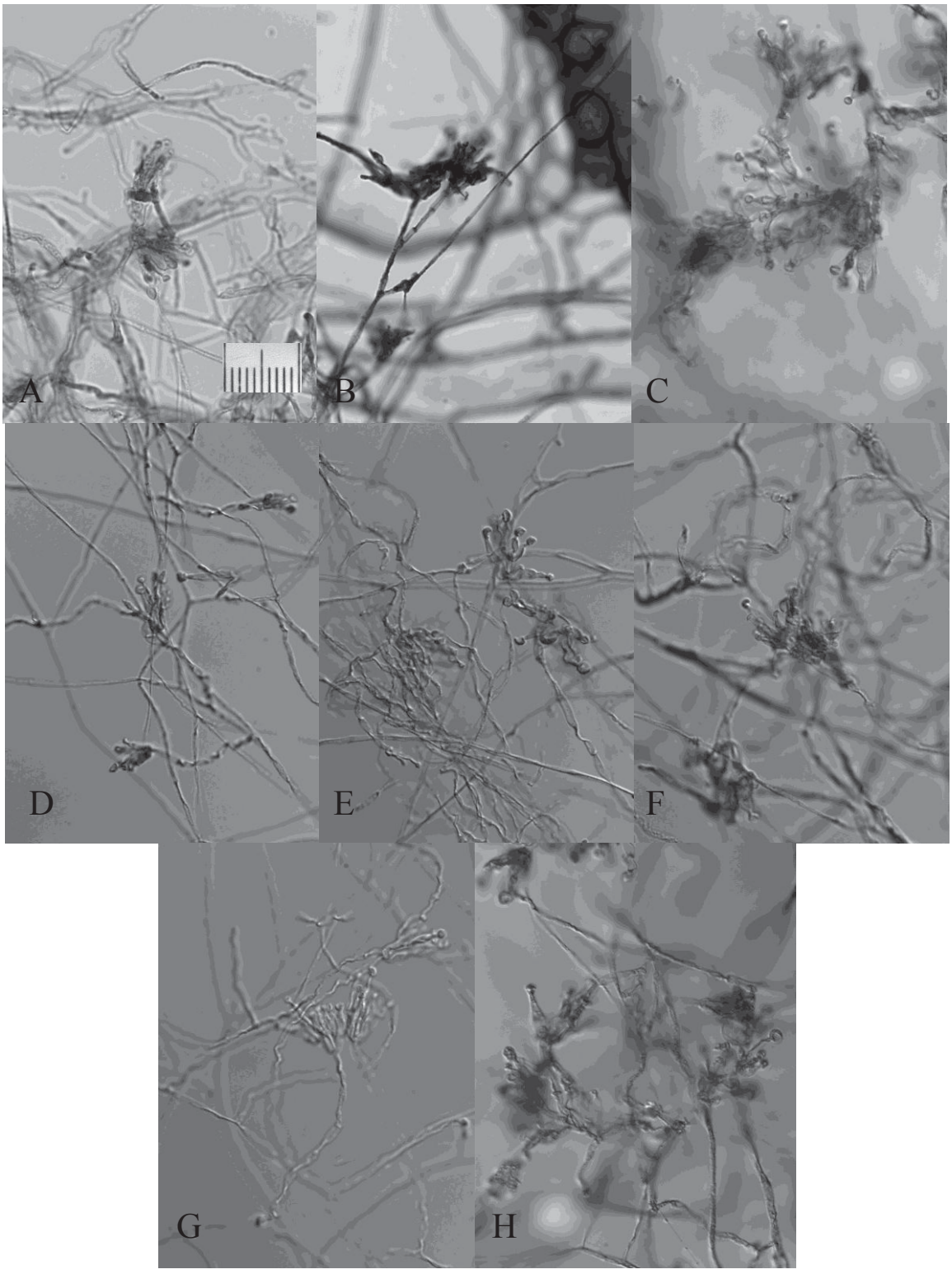

Figure 15. A- H) 100x magnification and scale bar represents 0.1 mm; *P. marneffei aspC* mutant CW slide culture assay; A) PDA only; B) 10 µM CW/ PDA; C) 20 µM CW/ PDA; D) 30 µM CW/ PDA; E) 40 µM CW/ PDA; F) 50 µM CW/ PDA; G) 60 µM CW/ PDA; H) 70 µM CW/ PDA.

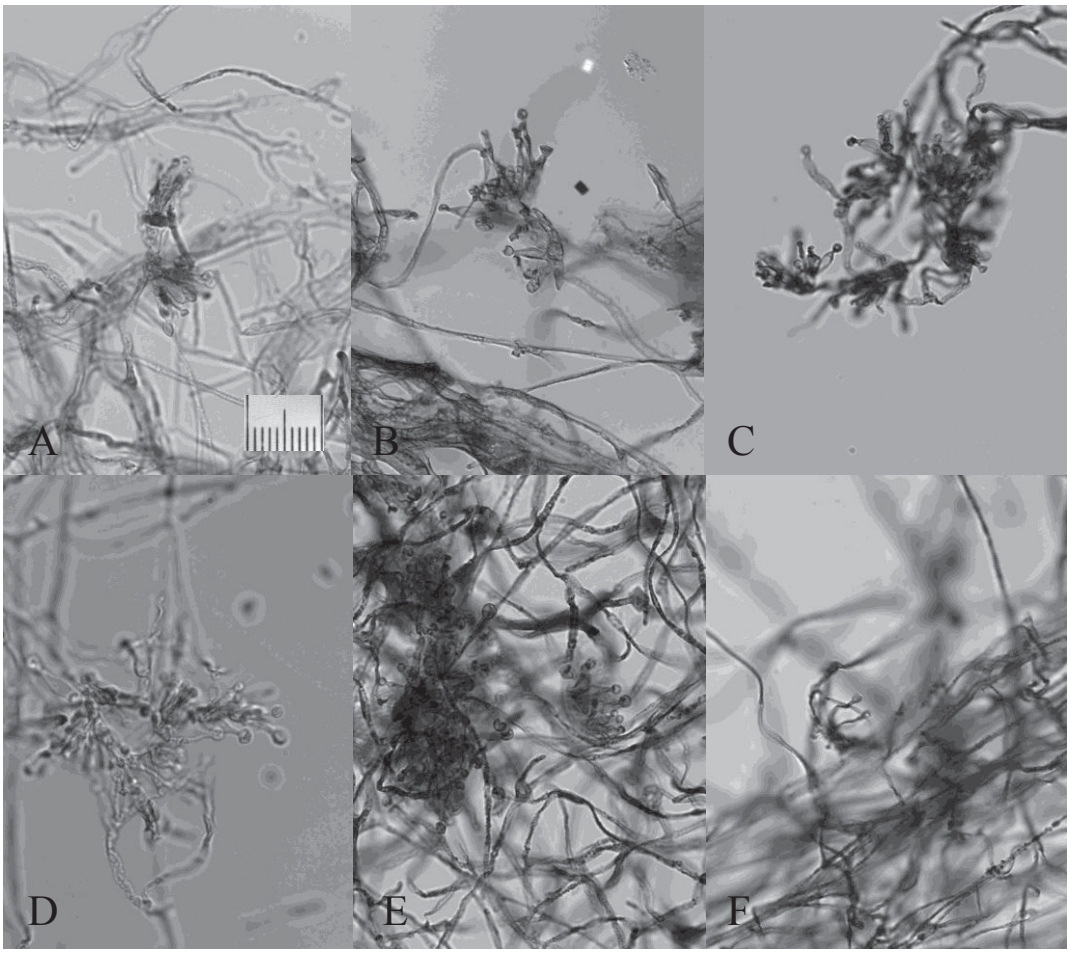

Figure 16. A- D) 100x magnification and scale bar represents 0.1 mm; *P. marneffei aspC* mutant H_2O_2 slide culture assay ; A) PDA only; B) 1 mM H_2O_2/ PDA; C) 1.5 mM H_2O_2/ PDA; D) 2 mM H_2O_2/ PDA; E) 2.5 mM H_2O_2/ PDA; F) 3 mM H_2O_2/ PDA.

Gene Expression Analyses

In order to quantitate expression levels of the *asp* genes, cDNA samples were subjected to qPCR. Expression levels were also to be considered significant only if expression was two- fold or higher than the expression levels observed in the mold phase of the wild-type strain cultured in SAB. If expression levels were less than two-fold greater, then those data were considered to be a trend but not significant.

After several qPCR experiments focusing on the expression of *aspA* in the wild-type and mutant strains, the corresponding results of each experiment revealed DNA contamination. Due to repetitive contamination and time constraints, expression of *aspA* was not characterized.

However, septin gene *aspC* was universally upregulated in the *aspC* mutant compared to the wild-type under every condition (Figures 19-26).

Under the described culture conditions of *P. marneffei* wild-type, *aspE* was frequently expressed at the highest levels, followed by similar expression levels between *aspD* and *aspB*, with *aspC* typically expressed the least (Figures 17, 19, 21, 23, 25).

Similarly, the *aspC* mutant also generally expressed *aspE* the highest (Figures 17, 19, 21, 23, 25). Unlike the wild-type strain, however, the *aspC* mutant frequently expressed *aspD* in greater levels than *aspB*. As observed in wild-type expression analysis, *aspC* was usually expressed the least.

Unexpectedly, higher expression levels of *aspC* were recorded in the *aspC* disruption mutant than were recorded in the wild-type strain in SAB- and MM- only

liquid cultures (Figure 19-20). The most extreme difference in *aspC* expression levels between mutant and wild-type was seen in SAB cultures incubated at 37°C, with about a 100-fold increase in expression in the mutant relative to the wild-type. In SAB cultures at both 25°C and 37°C, an increase in *aspD* expression levels was observed in the *aspC* mutant, while much higher expression levels of *aspD* were present in the mutant in MM cultures at both 25°C and 37°C. The greatest-fold difference in *asp* gene family expression was between the wild-type and mutant MM cultures incubated at 25°C. Expression levels of *aspC*, *aspD*, *aspE*, and *aspB* in the mutant were about 10-fold, 70-fold, nine-fold, and four-fold, respectively, greater than those recorded in the wild-type.

Treatment of cultures with H_2O_2 lead to an increase in expression levels of each *asp* gene under every condition (Figure 21). *aspD* was expressed the greatest in SAB and MM cultures incubated at 37°C, followed by *aspC* in the SAB culture and *aspE* in the MM culture. Again, *aspE* continued to be highly expressed.

Expression levels of the *asp* gene family in CR cultures were higher in the *aspC* mutant than in the wild-type under nearly every condition, with the exception of *aspB* in SAB and MM cultures incubated at 37°C (Figure 23). *aspC* was expressed the least under every condition except for in the *aspC* mutant cultured in SAB at 37°C, under which *aspC* was expressed about 50-fold greater than in its wild-type counterpart. Again, *aspE* was expressed the most.

With the exception of *aspE* in SAB cultures incubated at 37°C, all *asp* genes were more highly expressed in the *aspC* mutant than in the wild-type in CW cultures (Figure 25).

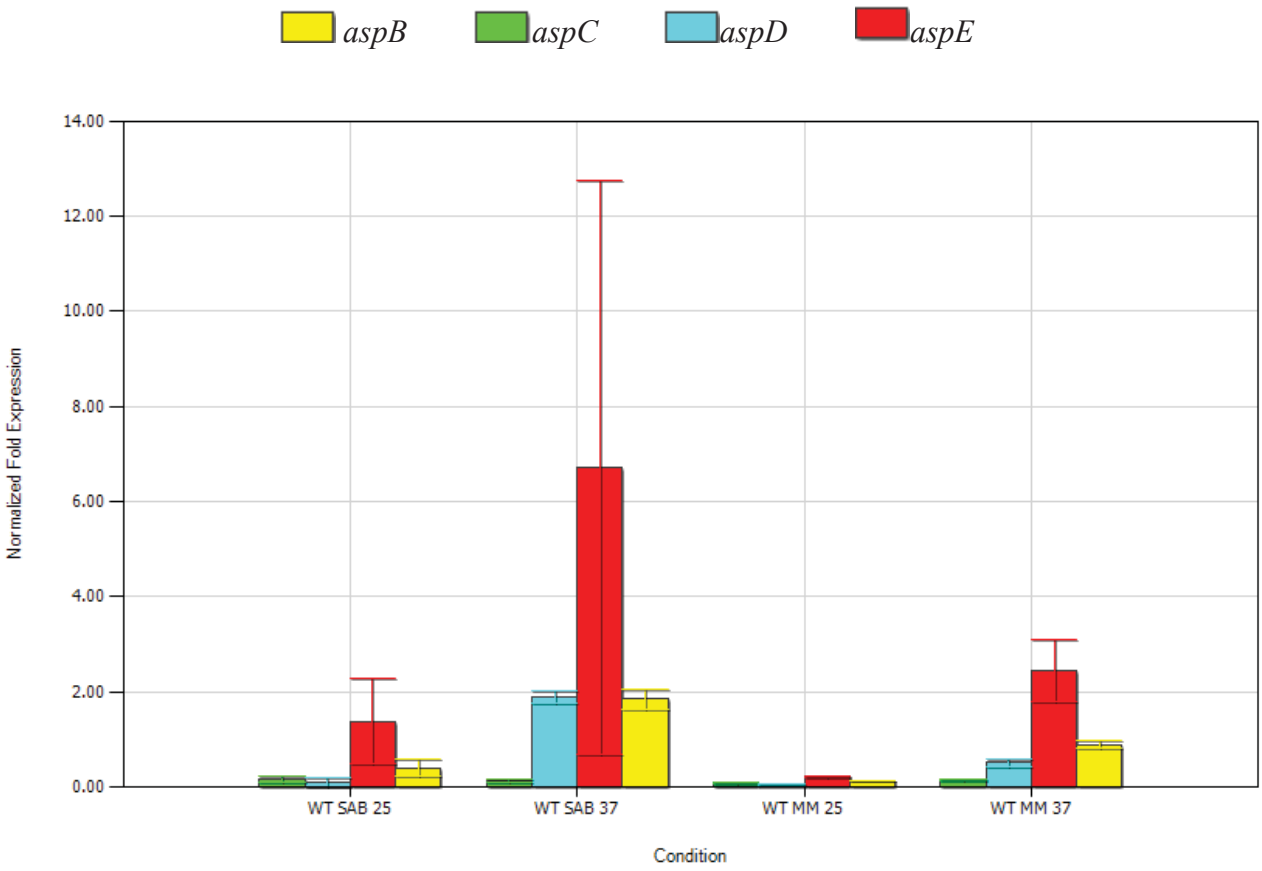

Figure 17. Differential expression of *aspB*, *aspC*, *aspD*, and *aspE* in *P. marneffei* wild-type cultured in SAB and MM incubated for two days at 25°C and 37°C.

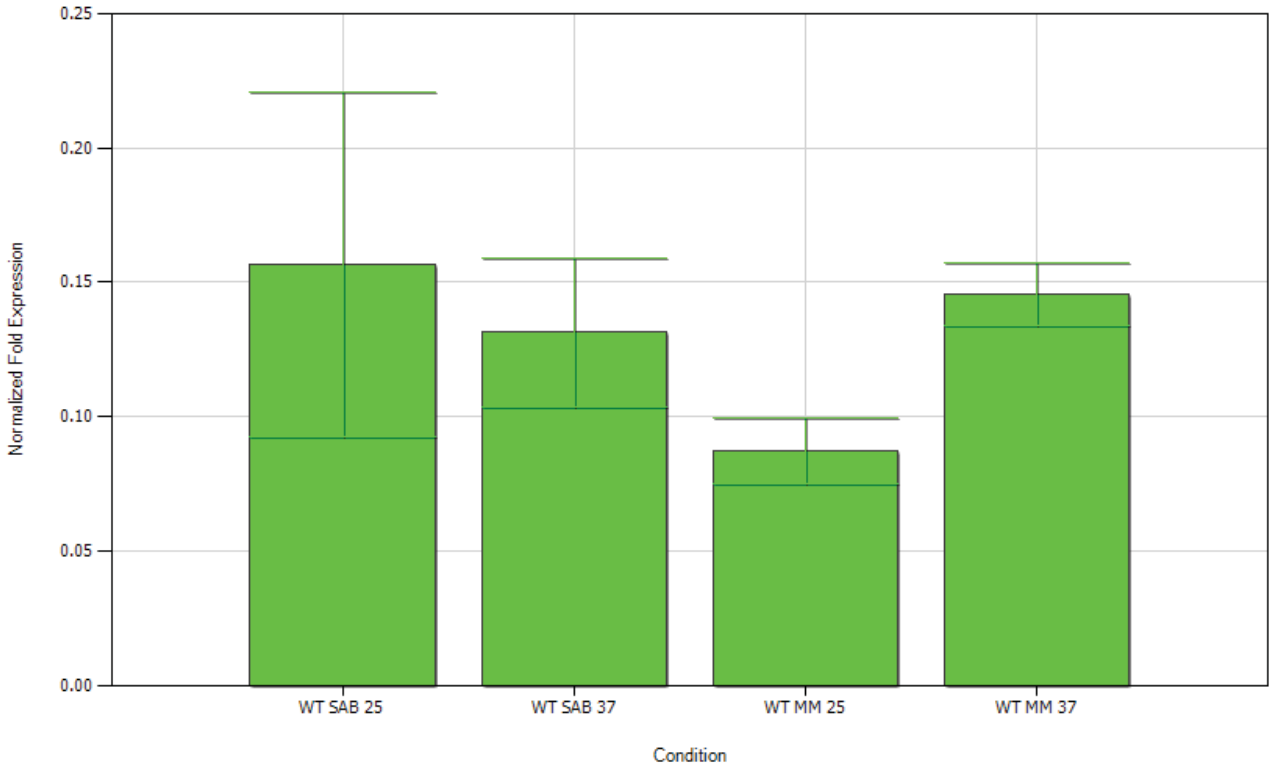

Figure 18. Differential expression of *aspC* in *P. marneffei* wild-type cultured in SAB and MM and incubated for two days at 25˚C and 37˚C.

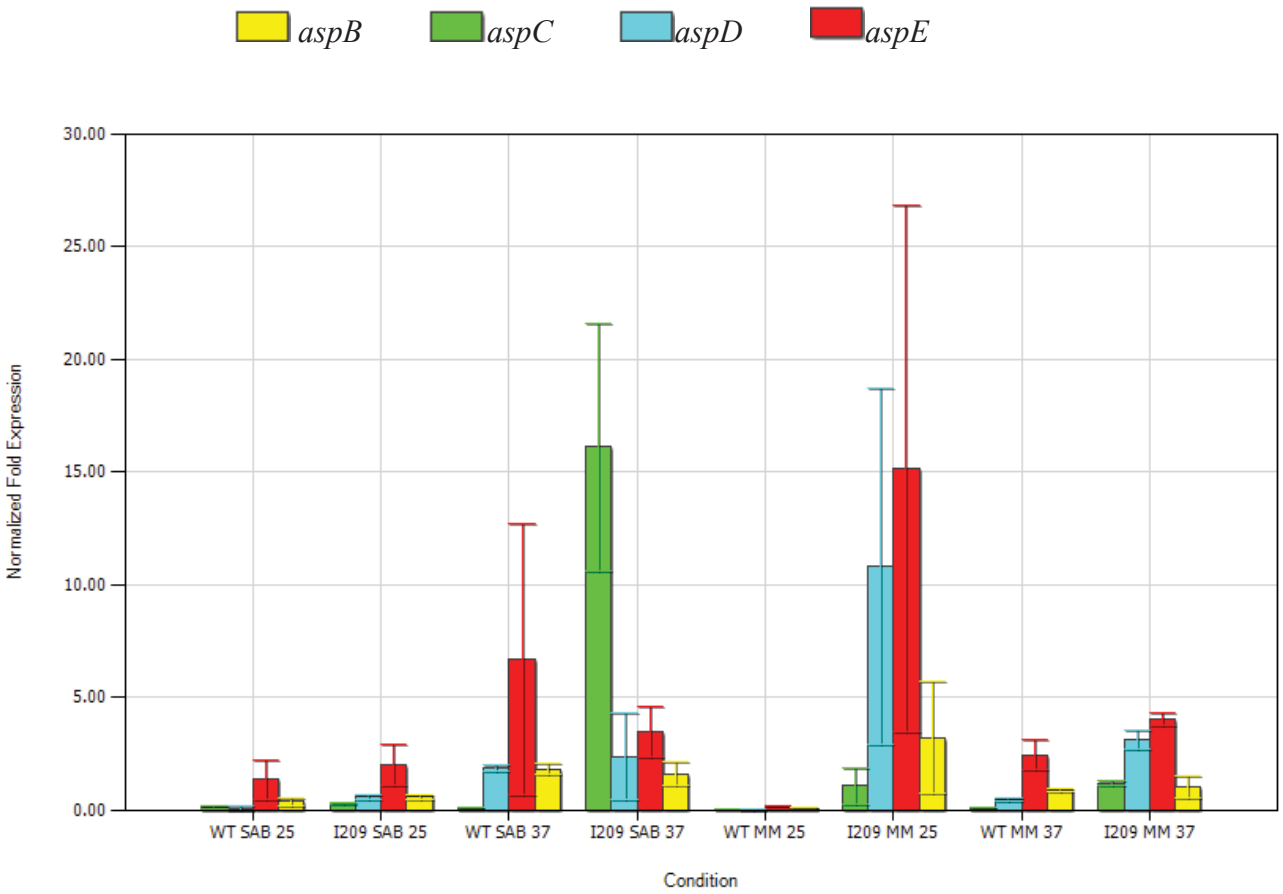

Figure 19. Comparison of differential expression of *aspB*, *aspC*, *aspD*, and *aspE* in *P. marneffei* wild-type and *aspC* mutant cultured in SAB and MM incubated for two days at 25°C and 37°C.

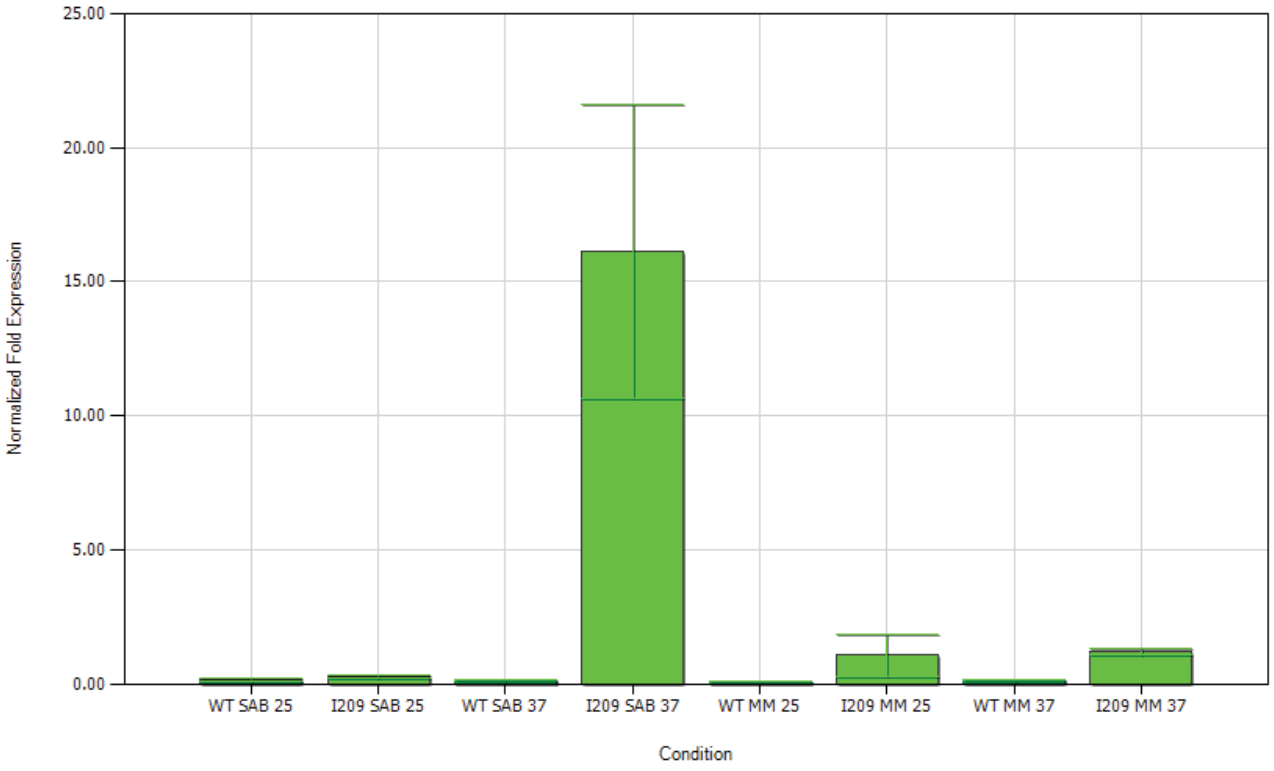

Figure 20. Differential expression of *aspC* in *P. marneffei* wild-type and mutant strains cultured in SAB and MM incubated for two days at 25°C and 37°C.

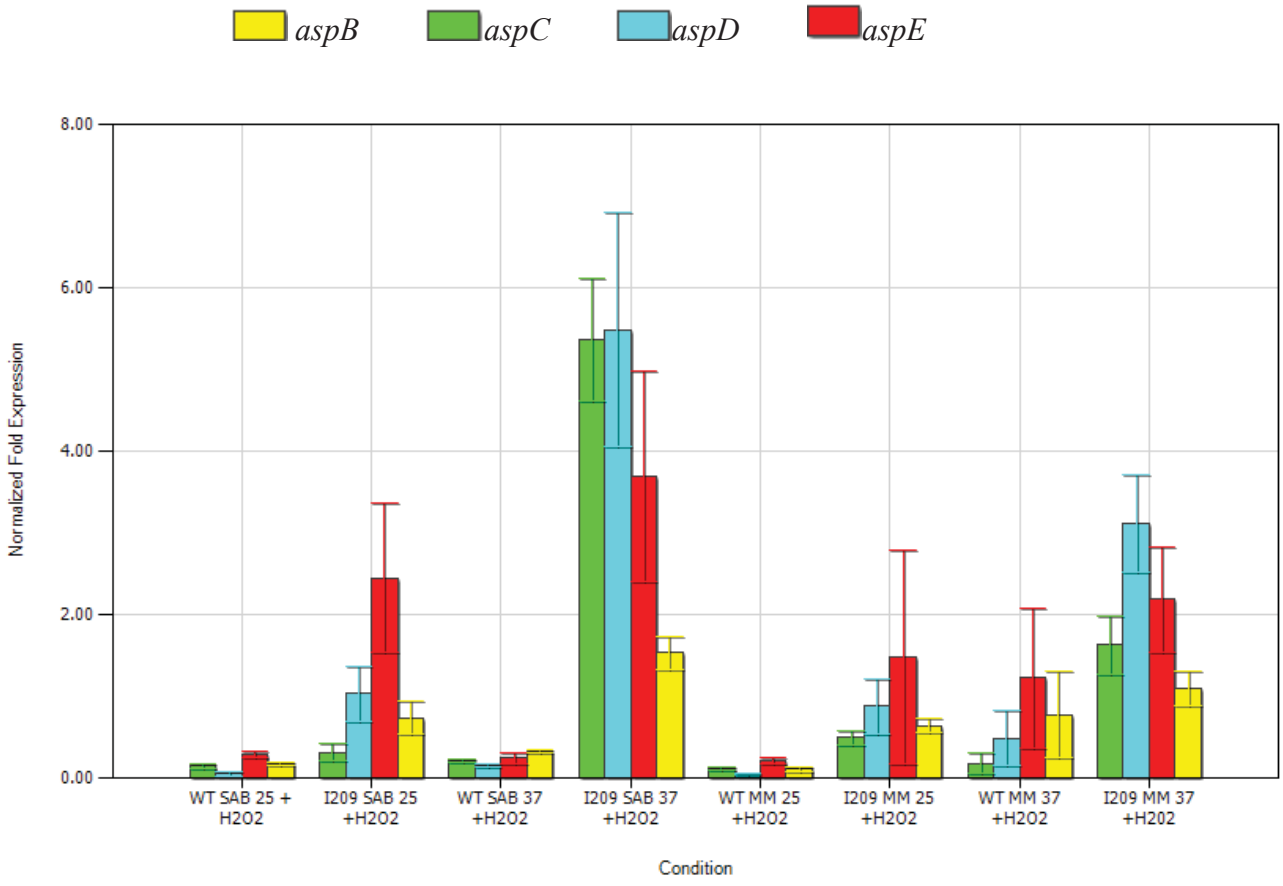

Figure 21. Differential expression of *asp* genes (*B*, *C*, *D*, and *E*) in *P. marneffei* wild-type and *aspC* mutant cultured in SAB and MM supplemented with H_2O_2 incubated for two days at 25°C and 37°C.

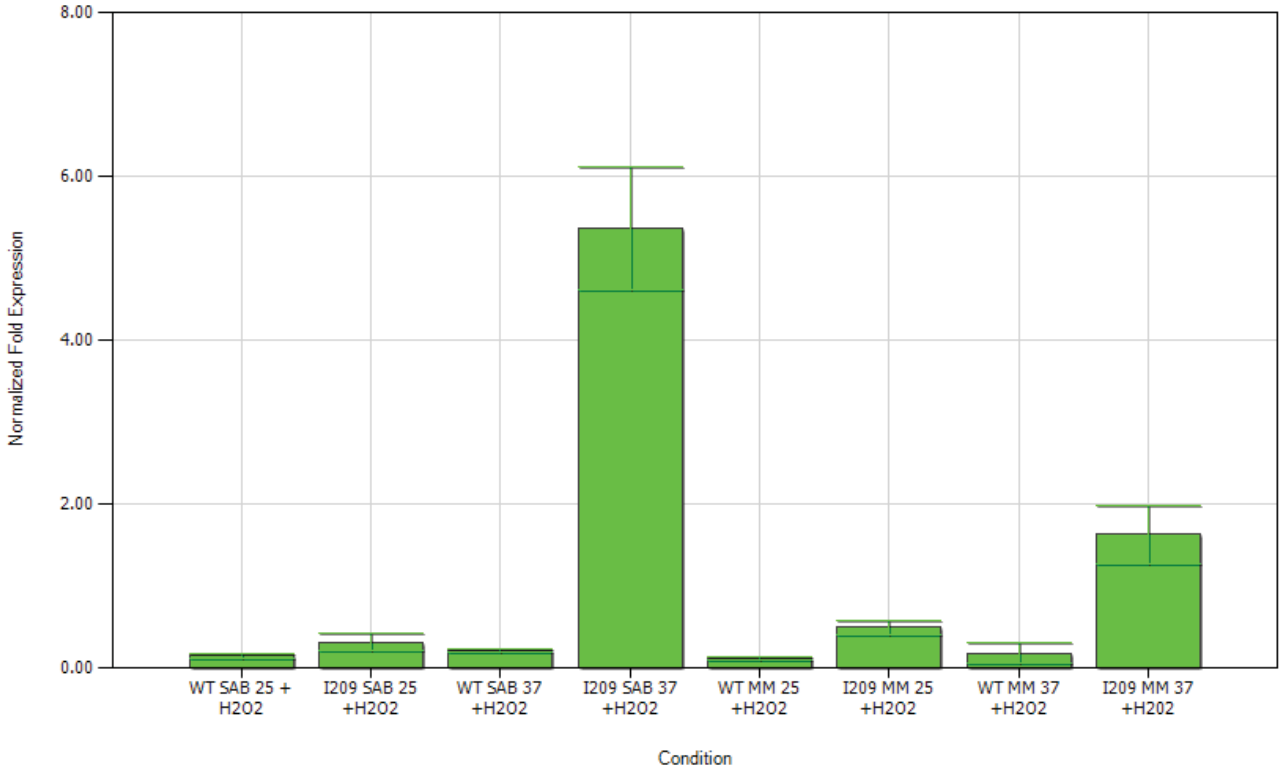

Figure 22. Differential expression of *aspC* in *P. marneffei* in wild-type and mutant strains cultured in SAB and MM supplemented with H_2O_2 incubated for two days at 25°C and 37°C.

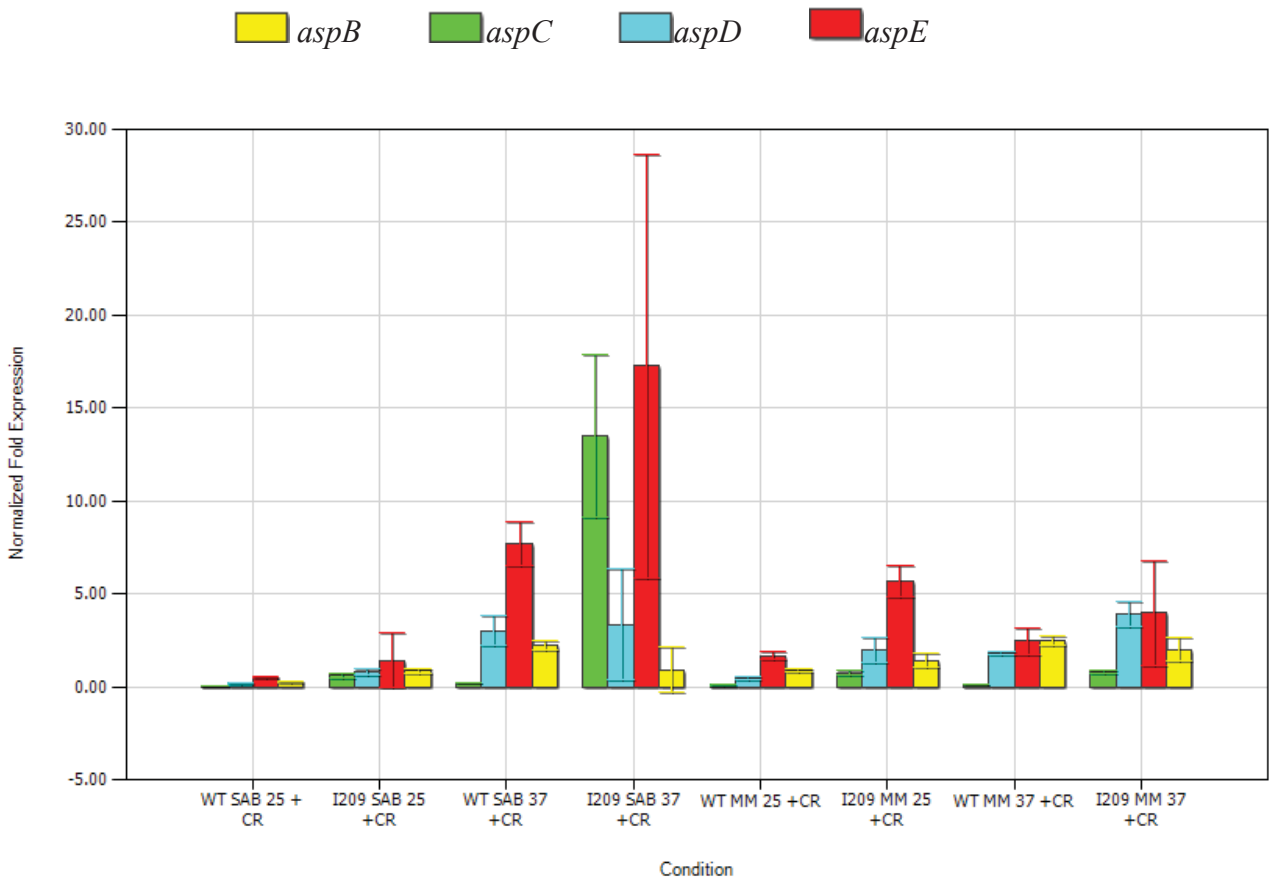

Figure 23. Comparison of differentially expressed *asp* genes (*B*, *C*, *D*, and *E*) between *P. marneffei* wild-type and *aspC* mutant cultured in SAB and MM supplemented with CR and incubated for two days at 25°C and 37°C.

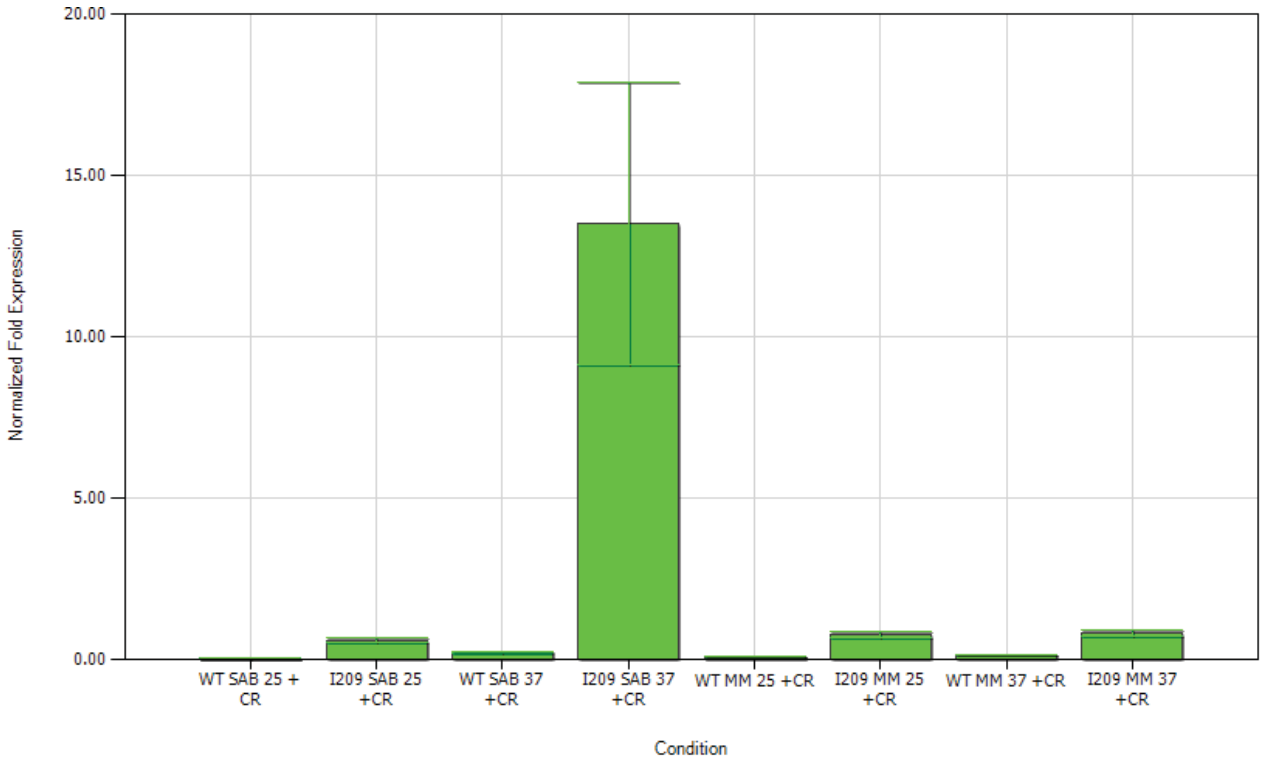

Figure 24. Differential expression of *aspC* in *P. marneffei* wild-type and mutant strains cultured in SAB and MM supplemented with CR incubated for two days at 25°C and 37°C.

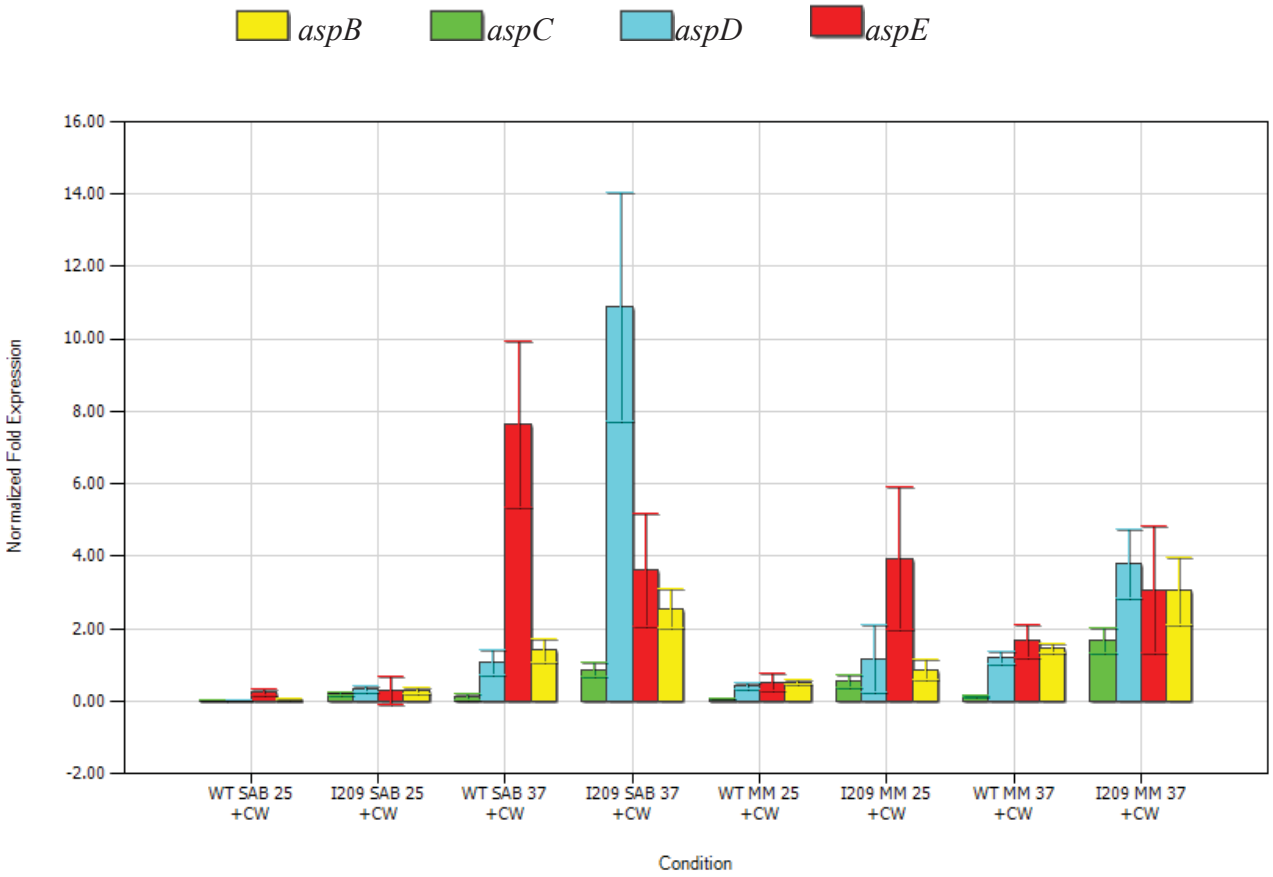

Figure 25. Differential expression of *aspB*, *aspC*, *aspD*, and *aspE* in *P. marneffei* wild-type and *aspC* mutant cultured in SAB and MM supplemented with CW incubated for two days at 25°C and 37°C.

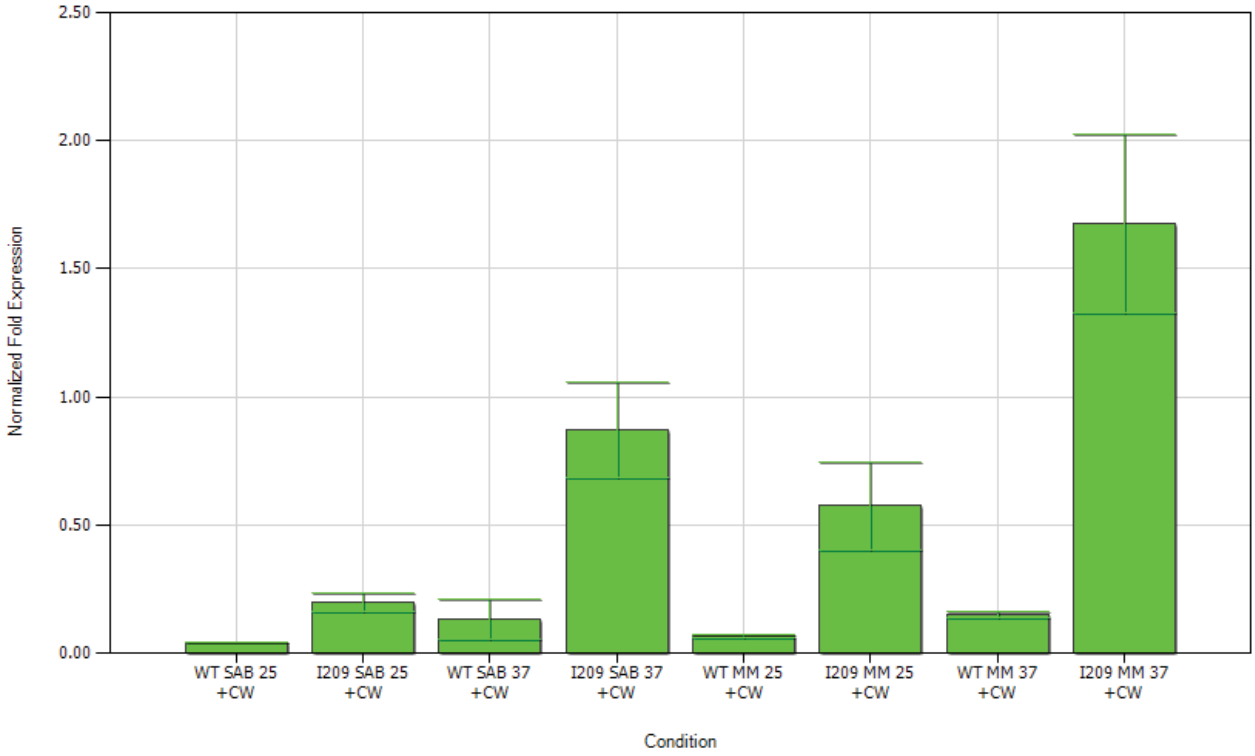

Figure 26. Differential expression of *aspC* in *P. marneffei* in wild-type and mutant strains cultured in SAB and MM supplemented with CW incubated for two days at 25°C and 37°C.

Chapter IV

Discussion

Microscopic Analysis

Microscopic analysis of wild-type and *aspC* mutant *P. marneffei* strains revealed profound differences in the gross anatomy of asexual structures. The lack of completely developed conidiophores and extensive chains of conidia observed in *aspC* cultures suggests an inability to complete the budding process that produces the chains of conidia found in wild-type cultures. Because the *aspC* mutant produces phialides that appear to stop budding after one conidium is produced, it may stand to reason that *aspC* is involved in conidiation and the remaining functional septins are compensating for the mutated gene to the extent of producing a single conidium but are not entirely adequate for proper conidiation. Alternatively, although, perhaps less likely, the presence of conidia in *aspC* mutant cultures may mean that *aspC* is not directly involved in conidiation in *P. marneffei*, but either plays a limited role in conidiation or is more directly involved in some process upstream of conidiation in the asexual developmental program, and the other septins are compensating for the mutated *aspC* or do not function properly without the effects the *aspC* product, respectively. Additionally, because conidia bud basipetally, new cell wall material needs to be produced at the conidium- phialide interface. If the *P. marneffei aspC* is involved in conidiation, then a mutation in *aspC* may lead to incomplete mitosis, which, in large part requires expansion and subsequent contraction of the cell wall and cell wall associated constituents, potentially yielding the abnormal conidiation pattern observed in the *aspC* mutant.

Sensitivity Assays

Sensitivity assays revealed differences both in the general growth rate and the sensitivity levels of each strain to various cell wall disrupting agents. The growth rate of the *aspC* mutant was markedly slower than that of the wild-type in negative controls.

After comparing H_2O_2 affected wild-type and mutant strain colonies to those cultured on the negative control plates, it was obvious that growth of both wild-type and mutant strains was inhibited at the highest concentration of H_2O_2. However, H_2O_2 presumably does not inhibit fungal growth via interference with the cell wall. H_2O_2 has been shown to cause an increase in ROS inside fungal mitochondria, leading to the degradation of the mitochondrial membrane potential. In turn, mitochondrial proteins involved in respiration are dysfunctional and the cell consequently dies. Since both strains were noticeably affected by H_2O_2, *aspC* may not be involved in the protective response to H_2O_2.

As previously described, CR binds chitin in such a way that chitin cannot properly form the larger crystalline networks observed in untreated hyphae. When considering the proposed mechanism and documented effects of CR-chitin binding, it should also be noted that septins serve in a variety of capacities, including the recruitment of proteins involved in chitin biosynthesis and localization (DeMarini et al. 1997; Kozubowski et al. 2003; Sanz et al. 2004). In *S. cerevisiae*, CDC10, the AspD homologue, has been shown to recruit Bni4, a scaffolding protein involved in cell wall biosynthesis, to the cell wall on the mother's side of the bud site (DeMarini et al. 1997; Kozubowski et al. 2003). Then, possibly in a concerted mechanism, Bni4 interacts with the activating sub-unit of chitin

synthase III (CSIII), Chs4, which interacts with Chs3, the catalytic sub-unit of CSIII (Sanz et al. 2004). Additionally, analyses of Bni4 deletion strains have shown that Bni4 is essential in localizing Chs4 but not necessary for CSIII activity, although Bni4 is involved in anchoring Chs3 to the neck region during bud emergence. These events contribute to process of chitin synthesis and deposition as a whole. In the case of the *aspC* disruption mutant, a dysfunctional AspC may be present in the septin complex-cell wall interface. A dysfunctional AspC may, in turn, lead to a partial failure in recruiting Bni4 to the cell wall. Hence, chitin synthesis and deposition in the cell wall would be compromised, and the *aspC* mutant's growth would be inhibited at higher concentrations of CR.

Similar to CR, CW binds chitin and interferes with proper crystallization of chitin in the fungal cell wall. However, no difference in growth was detected between *P. marneffei* wild-type and mutant strains in the CW assay, which suggests that the range of concentrations used in the assay was too low and that *aspC* is possibly not involved in cellular response to CW.

***asp* Gene Expression**

aspC Expression. The *aspC* mutant strain cultured in SAB at 37°C expressed *aspC* nearly 100-fold higher than the wild-type counterpart. This observation may have two explanations, possibly occurring in tandem. In the case of the difference in *aspC* expression between the mutant and wild-type strains cultured in SAB at 37°C, it is possible that the *aspC* mutant is recognizing that a dysfunctional AspC is being translated from a transcript of the mutated *aspC* and is continually signaling that the transcription

process for *aspC* needs to be repeated in order to replace the dysfunctional AspC with a functional version, causing a dramatic increase in mutant *aspC* transcripts. However, due to the disrupted *aspC* gene, only aberrant *aspC* transcripts are produced. This process may be inadvertently resulting in a feed-back loop. Because qPCR quantitates the amount of DNA being transcribed (i.e., transcripts) from cDNA in real time, the software associated with the thermal cycler may be recording the cells' homeostatic response to correcting an error in its translation of the *aspC* transcript. This analysis of *aspC* also applies to the general pattern of expression of *aspC* in the *aspC* mutant.

The explanation of differential *aspC* expression between the mutant strain cultured in SAB at 25°C and 37°C may be less convoluted. *P. marneffei*'s dimorphic nature causes increased amounts of cell wall reconstruction. Because septins are so extensively involved in cellular processes that affect cell wall shape and construction, the phase transition program may lead to the increase in *aspC* transcription. However, because the dimorphic switch was not inhibited, it is not likely that *aspC* is required for *P. marneffei*'s dimorphic switch.

Similarly, in *aspC* mutant SAB cultures supplemented with H_2O_2 incubated at 37°C, *aspC* levels were at least 37-fold greater. This discrepancy in *aspC* levels may again be explained by the presence of a dysfunctional AspC leading to repeated transcription of *acpC*. However, since these cultures were treated with a chemical that is known to effect the overall growth and development of cells (Qin et al. 2011), it may also stand to reason that *aspC* is involved in the reaction to protect the cell against the effects of H_2O_2 exposure.

Expression levels of *aspC* were also about 50-fold higher in mutant SAB cultures supplemented with CR at 37°C. Again, the previously proposed hypothesis for *aspC* expression in the *aspC* mutant may explain the recorded *aspC* expression levels in the mutant strain. In addition, as previously described, CR causes direct cell wall stress. The combined effects of the dysfunctional AspC and CR could be a likely cause of the increase in *aspC* expression.

aspE Expression. The *aspC* mutant expressed *aspE* at a higher level under nearly every tested condition. In *A. fumigates*, AspE is thought to play roles in both septation and intracellular transport. The up-regulation of *aspE* of more than four-fold in wild-type SAB cultures at 37°C can possibly be attributed to the beginning stages of phase transition, during which the cell wall and associated cytoskeleton undergoes tremendous changes, including polymerization of cell wall constituents, transport of enzymes to the cell wall, transport of cellular organelles to new hyphal branches, etc. If *aspE* were actually involved in intracellular transport, then it would be reasonable to suspect that *aspE* should be up-regulated in the wild-type at 37°C. In the *aspC* mutant, an increase in *aspE* expression levels may also be explained by the cell up-regulating *aspE* to compensate for a dysfunctional AspC, in addition to the possibly increased role of *aspE* during phase transition.

After supplementation with H_2O_2, minor decreases were also recorded in the wild-type strain cultured in SAB at 25°C and 37°C, respectively, as well as in MM at 37°C. Conversely, there was about a two-fold increase of *aspE* observed in the mutant strain cultured in SAB at 37°C. Because H_2O_2 is known to oxidatively stress cells via protein

denaturation (Qin et al. 2011), it is possible that some proteins involved in transcription, either at the nuclear or cytosolic signaling levels, were in some way inhibited.

Differential expression analysis of *aspE* after CR exposure revealed an increase in expression of *aspE* in every condition. An approximate 12-fold increase in *aspE* expression in the mutant strain cultured in SAB at 37°C was observed. Additionally, more than a five-fold increase in *aspE* expression was recorded in the wild-type strain cultured in SAB at 37°C. These fluctuations in *aspE* suggest that the gene may play a role in the cell's response to the effects of CR.

The expression levels of *aspE* in both strains cultured with CW were inconclusive. Interestingly, though, a significant increase in *aspE* was evident in the wild-type strain cultured in SAB at 37°C.

aspD Expression. *aspD* was expressed in a greater amount in the *aspC* mutant than the wild-type in every instance. In addition, up-regulation of *aspD* was recorded in the wild-type strain at 37°C in every instance and nearly every condition in the mutant strain. These results suggest that *aspD* plays a role in phase transition.

After exposure to H_2O_2, expression of *aspD* was approximately 50-fold higher in the *aspC* mutant cultured in SAB at 37°C. While *aspD* appears to be involved in phase transition, it is inconclusive as to whether *aspD* is involved in the response to H_2O_2. Results also suggest that the expression of *aspD* is also inhibited by the presence of H_2O_2.

With the exception of the *aspC* mutant cultured in MM at 25°C, there was essentially little difference in the expression of *aspD* in the wild-type and mutant strains

cultured with and without CR. These results suggest that *aspD* is not necessarily involved in the response to CR.

Treatment with CW promptly lead to an approximate 110-fold increase of *aspD* in the *aspC* mutant cultured in SAB at 37C. *aspD* expression also increased in the mutant cultured in MM at 37°C, with nearly a 40-fold increase. These results potentially provide further evidence that *aspD* is not necessarily involved in the response to CW, but possibly phase transition.

aspB Expression. While expression levels of *aspB* did fluctuate, the largest-fold difference was a seven-fold increase in the mutant strain cultured in MM at 37°C. Because *aspB* has been documented as being primarily involved in septation, the expression levels of *aspB* were lower than expected.

In addition, while not necessarily significant in every case, *aspB* expression did increase in the yeast phases of both the wild-type and mutant strains, suggesting a role for *aspB* in dimorphism.

While H_2O_2 and CW did have a small but noticeable effect on *aspB* expression, CR did not appear to have an effect. These results suggest that *aspB* may not be involved in the responses to cell wall stress via polysaccharide binding dyes or the response to oxidative stress.

In conclusion, these preliminary results, taken together, suggest that *aspC* plays little to no role in phase transition in *P. marneffei*. However, the increased levels of *aspE* suggest the AspE septin may be involved in phase transition. Further *asp* gene family analyses will be required to state the significance of each septin in *P. marneffei*. In

addition to differential gene expression analyses, experimentation focusing on possible colocalization of septins with cytoskeletal elements, such as microfilaments, microtubules, and thin filaments, should be conducted concomitantly to elucidate the relationship between septin function and morphology.

Chapter V

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