LIPID BIOSYNTHESIS AND MATURATION SIGNALS IN *BATRACHOCYTRIUM DENDROBATIDIS* IN VITRO

BY

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ABBREVIATIONS

*Bd*: *Batrachochytrium dendrobatidis*

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

ANOVA- analysis of variance
**ABSTRACT**

*Batrachochytrium dendrobatidis (Bd)* is a fungal parasite of amphibians responsible for the disease chytridiomycosis and partly attributed to massive global population declines of frogs and salamanders. Many of the molecular mechanisms behind the growth and development of *Bd* and how the fungus invades and infects amphibian hosts are unknown. In this study, two aspects of *Bd* development were analyzed. Confocal microscopy was used to observe lipid biosynthesis and packaging throughout the sessile life stage of the fungus. Maturation assays were conducted to examine the density-dependent maturation of *Bd* and to isolate potential developmental regulators secreted by zoosporangia that quicken the development of conspecifics. In addition, the isolation of proteins responsible for host-invasion was attempted using SDS-PAGE. Results from maturation assays support that multiple secretory molecules work synergistically to quicken maturation of zoosporangia. Further isolation of the responsible agents will give insight into how *Bd* development is regulated in the field. While the lipid analysis results were inconsistent, the data suggest a possible increase in lipid stores as sporangia mature, followed by packaging of lipids into newly forming zoospores in both cell membranes and energy stores. Bands from the protein extraction for invasion mechanism determination were not sufficient for analysis.
Chapter 1: Introduction to chytridiomycosis and recent research on

Batrachochytrium dendrobatidis

Starting in the 1980s, herpetologists and ecologists began noticing the absence or decline in many numbers of amphibian populations at field sites around the world (Collins and Storfer, 2003). Since then, vast work has been done to determine the cause(s) of these local extinctions and population declines and, in 1998, Batrachochytrium dendrobatidis (Bd), a fungal parasite of amphibians, was first described and linked to those extirpations (Berger et al., 1998). The fungus causes the disease chytridiomycosis, which is characterized by lethargy, skin sloughing, loss of righting reflex, anorexia, and death (Nichols et al., 2001). Until recently, Bd was the only known member of the phylum Chytridiomycota that is a parasite of a vertebrate (Berger et al., 1998). This changed with the recent identification of Batrachochytrium salamandrivorans, a unique chytrid fungus that is expected to occupy a different environmental niche (Martel et al., 2013). Although not all species of amphibian are susceptible to infection by Bd, the population declines of over 200 species of frogs and salamanders have been partly attributed to chytridiomycosis (Skerratt et al., 2007).

Life Cycle

Bd has two distinct asexual life stages. The first of which is a motile zoospore with a single flagellum. This stage lacks a cell wall (Greenspan et al., 2012). Zoospores exhibit positive chemotaxis towards keratin, sugars, amino acids, and proteins, which suggests a possible mechanism of host location within a body of water (Moss et al., 2008). Zoospores are usually motile for approximately 24 hours before adhering to a substratum,
retracting the flagellum, and laying down a chitinous cell wall. Outside of a host, the zoospore develops into a single or colonial zoosporangium with rhizoids (Greenspan et al., 2012). The ability of *Bd* to be cultured indicates that it could grow saprophytically in nature when in absence of a suitable host (Longcore et al., 1999). When adhered to a host, a germination tube forms from the encysted zoospore (now called a thallus) and extends intracellularly, often through multiple host cell layers. Contents of the thallus then travel through the germination tube into the host tissue, forming a bulge on the receiving end, which develops into a zoosporangium. A septum forms separating the newly forming sporangium from the germination tube. When the superficial layer of amphibian skin sloughs off, the empty thallus and germination tube fall away, leaving the intracellular zoosporangium intact (Greenspan et al., 2012). Infected frog skin shows hyperkeratinization and excessive sloughing that leads to the exposure of incompletely keratinized cells. Local cytoplasmic condensing and cellular content displacement can occur near intracellular thalli and sporangia, creating clear zones (Greenspan et al., 2012, Berger et al., 2005).

The contents of the zoosporangium (whether inside a host cell or on an experimental substratum) undergo mitotic divisions and cleave into new zoospores. The approximate number of spores produced per sporangium is unknown. Mature zoospores are released via an inoperculate opening through one, or more, discharge papillae that form per sporangium (Berger et al., 2005). Spores may be released to the environment, where they will search for another host, possibly through chemotaxis (Moss et al., 2008), or they may be released into intracellular spaces, where they will further infect the host.
Life cycle duration of sporangia varies between *Bd* strains and is affected by other factors, such as temperature (Berger et al., 2005, Voyles et al., 2012).

*Bd* invades the keratinized cells of the mouthparts of tadpoles and the epidermis of adult frogs. The localization to the mouthparts of tadpoles limits the spread of infection and inhibits mortality (Berger, et al. 1998); however, infection in tadpoles decreases development rate, possibly through reduced foraging efficiency (Venesky et al., 2009). Why *Bd* requires keratinized tissue of amphibians for infection is unknown. Though *Bd* invades keratinized tissues, its intracellular growth inside the host occurs in deeper layers of the epidermis that contain prekeratin, and much of sporangial development occurs before the infected cell becomes completely keratinized (Berger et al., 2005).

**Disease Progression and Means of Host Resistance**

There are two possible explanations for the mortality associated with *Bd* infection, i.e., the fungus may release proteolytic enzymes or other compounds, which may be absorbed through the host’s skin, and infection may disturb skin function and disrupt electrolyte balance. The latter explanation is supported by work done by Voyles et al. (2009). Electrolyte absorption is highly regulated in amphibians to maintain an internal environment that is hyperosmotic relative to the external environment (Voyles et al., 2011). This regulation involves Na⁺/K⁺ pumps and sodium channels in the epidermis. Electrolyte transport (primarily sodium absorption) across the skin is lower in diseased frogs. Whether this disruption is due to direct physical damage or a toxin secreted by the fungus is unknown. In addition, sodium and potassium concentrations in the blood are
reduced in infected frogs. Because body mass and total protein concentrations in the blood do not change in infected frogs compared to control frogs, observed osmotic imbalances are likely due to electrolyte loss and not simply dilution due to increased water uptake. Prior to the death of infected frogs, cardiac electrical activity resembles asystolic cardiac arrest, which normally occurs due to contractile failure caused by electrolyte abnormality, further supporting that mortality in chytridiomycosis is due to disrupted electrolyte balance. Moreover, oral supplementation of electrolytes aids the recovery of normal posture and activity level in frogs in late stages of infection, although these frogs still die (Voyles et al., 2009).

Although chytridiomycosis is often a fatal disease, susceptibility to Bd infection varies across amphibian species. While some species experience severe infections and death, others can tolerate low levels of infections or can completely clear themselves of the fungus (Daszak et al, 2004). Many studies have focused on this variability, and the roles of innate and acquired immunity in susceptibility are still unclear and perhaps host-species-dependent.

Frogs secrete antimicrobial peptides (AMPs) from granular glands that aid in the nonspecific inhibition of pathogens on their skin. To address the potential function of innate immune responses against Bd infection, multiple AMPs from the skin of different frog species were tested and shown to be effective in inhibition assays against Bd (Rollins-Smith et al., 2002). Because different species of amphibians secrete different types and amounts of AMPs, these innate variations could be responsible in part for the differing levels of natural susceptibility or resistance to Bd across species. A study conducted using “hybrid advantage” regarding AMPs showed that hybrids of Pelophylax
lessonae and P. ridibundus are more effective at inhibiting Bd growth than either parent due to an additive effect in the range of AMPs produced and secreted (Daum et al., 2012). In addition, AMP depletion assays showed increased Bd susceptibility in the normally resistant Xenopus laevis on skin peptide depletion using norepinephrine (Ramsey et al., 2010). In another study, the numbers of granulocytes in the blood have been shown to increase upon infection with Bd, solidifying a role for innate immunity in chytridiomycosis resistance (Woodhams et al., 2007).

Another factor possibly aiding in the resistance of some amphibian species is the synergy of these AMPs with metabolites produced by the symbiotic microbiota of amphibian skin. One such metabolite from Pseudomonas fluorescens, 2,4-diacetylphloroglucinol (2,4-DAPG), is inhibitory to Bd growth. This species of bacteria is commonly found on Rana muscosa, where 2,4-DAPG was shown to work synergistically with AMPs from R. muscosa to inhibit the growth of Bd at reduced inhibitory concentrations. The variability in the microbiome, and thus bacterial metabolites present on the skin, of different species of amphibians living in different environments may be contributing to the variation in susceptibility that is seen in the field. The variability in bacterial metabolites and AMPs present may also serve as an obstacle in the evolution of resistance in Bd to these molecules (Myers et al., 2012).

When evaluating amphibian genomic responses of multiple tissues to infection with Bd, little or no acquired immune response in Rana muscosa and R. sierra was seen. In the skin, a decrease in expression of many immunity genes and an increase in anti-inflammatory markers were observed. In the liver, expression levels of immunity genes were generally unchanged, while immunity-related gene expression in the spleen
exhibited only an insubstantial and ephemeral cytokine signal-mediated response to *Bd* infection. CD4 expression was not elevated, indicating a lack of T-cell recruitment. However, there was increased gene expression of cathelicidens (antimicrobial proteins used in defense against fungal pathogens) and IL-1 (an inflammatory response marker). Even so, immune activation by *Bd* infection was very weak. As more functional gene annotations become available, further research will help expound on these findings. Further study is also needed to determine if the lack of acquired immune response is due to the lack of pathogen recognition or an active immune suppression by the pathogen (Rosenblum et al., 2012b).

In contrast to these findings, a study involving *Xenopus laevis*, a *Bd*-resistant frog species, revealed that inhibiting acquired lymphocyte function by irradiation caused greater infection intensity (higher number of zoospore equivalents present in the skin) in frogs compared to controls, although intensity declined to levels similar to the controls by day 44. Immunization studies were also performed on *X. laevis* using heat-killed *Bd*. This caused the induction of IgM and IgY antibodies against mature sporangia and zoospores that persisted for 28 days. Mucosal secretions were also tested for the presence of antibodies. Immunoglobulins from 3 different classes were found, and all 3 were capable of binding with *Bd* antigens. These data show that a specific, acquired immune response can be induced by killed *Bd* and that mucosal antibodies may aid in resistance against *Bd* in *X. laevis* (Ramsey et al., 2010). A similar study using formalin-fixed *Bd* in *R. muscosa* showed no effect of immunization on *Bd* infection or mortality when compared to that of control frogs immunized with saline (Stice and Briggs, 2010).
Disease survival or susceptibility in vertebrates is often associated with polymorphisms in Major Histocompatibility Complex (MHC) proteins. MHC Class II molecules are expressed on antigen-presenting cells; thus, they are intimately linked to T cell responses and the acquired immune responses to pathogenic peptides. To test the relationship between infection survival and genetic variation at MHC loci, the peptide-binding regions (PBR) of the MHC Class IIB genes of 99 frogs (*Lithobates yavapaiensis*) were characterized, and 33 unique PBR alleles were found. Both PBR heterozygosity and a specific allele, Q, were shown to be significant variables in predicting survival (Savage and Zamudio, 2011). These results also support a role for host acquired immunity in response to *Bd* infection. It is possible that further research will reconcile the conflicting findings described or will show that susceptibility to *Bd* is due to the loss of the immune responsiveness that is seen in the resistant species.

Another factor that seems to contribute to resistance to chytridiomycosis is the thickness of an amphibian’s skin. *Lithobates catesbeianus*, a species of frog resistant to chytridiomycosis, has more cell layers in the stratum granulosum and stratum spinosum than *Lithobates sylvaticus*, a species susceptible to mortal infection by *Bd*). The abnormal skin sloughing that chytridiomycosis causes in infected frogs may actually be beneficial to the host. This sloughing may serve to rid the frog of infected skin layers, thereby preventing re-infection by sporangia embedded in the skin. Excessive skin sloughing would become detrimental when electrolyte uptake is inhibited. The more numerous epidermal layers in *L. catesbeianus* may allow rapid enough sloughing to keep zoosporangia load low without being so severe that cutaneous function is harmed. Further work must be done on other resistant and susceptible species to determine if this
morphological difference in host epidermis consistently correlates with morbidity (Greenspan et al., 2012).

Climatic factors also play a role in chytridiomycosis resistance. Not only has temperature been shown to affect amphibian immune responses (Wright and Cooper, 1981), but it also affects Bd growth and morbidity. Bd grows optimally between 17-25°C. Amphibians with chytridiomycosis that are exposed to temperatures above this often clear their infections, and Bd cultures exposed to a suboptimal temperature of 4°C showed lower population growth rates but had longer zoospore activity periods (Voyles et al., 2012). For the reasons described above, response to infection is likely a combination of host, Bd strain, and environmental factors.

**Host Invasion Mechanism**

While Bd growth and development has been studied extensively at the morphological level, not much is known about its invasion mechanisms at the molecular and cellular level. Bd attaches to its substratum using mucilage. At this point, secreted proteases are likely involved in mediating invasion (Rosenblum et al., 2012a). Many pathogenic fungi use secreted peptidases, which are related to virulence. Common peptidases include the aspartyl-, serine-, and metalloproteases. Also used are aminopeptidases, carboxypeptidases, and dipeptidyl-peptidases (Monod et al., 2002). Metalloproteases, in particular, are expressed by many pathogenic fungi that, like Bd, infect vertebrates (Rosenblum et al., 2008). The latter study included whole-genome expression assays to examine gene expression and RNA abundance for the different life stages (zoospore and sporangia) of Bd under identical growth conditions using the Gene
Ontology database and InterPro for functional domains. These assays were conducted in vitro, using 1% tryptone in 1% agar plates. The results showed that over half of the genes in the genome were differentially expressed by the 2 life stages, with 1,522 of the 8,255 analyzed genes the same. Enrichment was observed for metalloexopeptidases in sporangia. When using less stringent gene sets for molecular function, ligase and helicase activity was enriched in zoospores and transmembrane transporter activity was increased in zoosporangia. (Rosenblum et al., loc. cit.)

When analyzing functional domains, fungalysin metalloproteases exhibit elevated expression due to gene-duplication in *Bd* and could be involved with keratin degradation or that of another cellular component (Rosenblum et al., 2008). Of the 25 fungalysin metalloprotease genes, 19 exhibited differential expression between sporangia and zoospore stage (18 of these being higher in sporangia). Also showing gene expansion in *Bd* were serine-type proteases. These enzymes may be involved in host substrate or peptide degradation against substrates like anti-*Bd* AMPs. Twelve of the 29 serine peptidase genes in the *Bd* genome revealed increased expression in sporangia. An increased expression in clathrin-related genes was also seen. This is notable because some viruses and bacteria use this gene family to enter host cells. These genes allow them to mimic ligands to the clathrin-dependent signaling system of the host. The gene expansion of aspartyl proteases in *Bd* also may be significant since they are used by other fungal pathogens in invasion and adherence to human tissue (Joneson et al., 2011). In addition to these protein families, several other genes that were differentially expressed revealed sequence similarity to immune system evasion genes of viruses and bacteria, e.g. ITAM and interleukin-1 (Rosenblum et al., 2008). These data show dramatic differences
between protein expression in the substrate-independent, and substrate-dependent, life stages of Bd and highlight the possibility of isolating the factors involved in actual penetration and invasion of amphibian skin.

Sixty-two genes in the Bd genome were found to encode Crinkler, or Crinkler-like, microbial effectors (Joneson et al., 2011). Prior to this discovery, these genes had only been found in oomycetes (protists once classified as fungi) and may have been acquired by Bd via horizontal gene transfer. Crinklers in oomycetes are intracellular effectors with C-terminal effector domains that are believed to enhance pathogenesis. Crinklers in Phytophthora capsici are thought to target the host nucleus and to reprogram the host to assist infection (Lamour et al., 2012). The function and expression of Crinklers in Bd needs to be studied, as they could play a role in Bd virulence similar to their role in oomycetes.

In addition to the differences in gene expression between zoospores and sporangia and the gene expansions seen in Bd, variation in virulence of the fungus could be attributed to proteomic differences between isolates. Regardless of low global genetic diversity (discussed below), Bd isolates are not identical, and much of current research regarding disease spread and host susceptibility does not account for the genotype of the particular infecting Bd strain. In a study comparing the protein expression of 6 different global isolates of Bd, 70 inter-isolate changes in expression were statistically significant. Forty-nine of these proteins were sequenced and were found to be associated with multiple cellular functions, including multiple proteins with proteolytic function and at least one that is a possible virulence factor in other eukaryotic pathogens. When 3 of
these global isolates were assessed for virulence, differences were seen in both pathogenicity and subsequent host mortality, indicating that Bd strain impacts virulence in the field (Fisher et al., 2009).

**Origin of Bd and Chytridiomycosis**

Why Bd has only recently emerged as a serious amphibian pathogen is still unknown. Hypotheses for this observation include recent climate change favoring Bd pathogenesis and the dissemination of Bd into novel host populations through natural or anthropogenic reservoir movement. A study comparing 17 polymorphic nuclear loci from 59 strains of Bd isolated from 31 host species from 5 continents showed no host-specificity of pathogen genotypes. Population structure of the isolates suggested that differences were due to population differentiation of the fungus that occurred after 1, or just a few, introductions to new regions. As much allelic diversity was seen in 1 specific isolate as was seen in the entire global sample. This study supports the hypothesis that Bd’s emergence as a global pathogen is due to the introduction of Bd into novel species rather than climate or environmental change (James et al., 2009).

Also supporting the notion that the Bd epidemic is due to a rapid range expansion is a recent multilocus sequence typing study of 10 gene regions from 35 Bd strains from North America, Central America, Africa, and Australia. The study showed that only 5 sites in the 10 loci were variable. This low level of genetic variation would not be as likely if the disease emerged due to environmental change and supports the hypothesis that the emergence is due to the recent introduction of Bd into novel populations. The
low-host specificity of *Bd* as well as the high mortality associated with infection further support this hypothesis (Morehouse et al, 2003).

The ultimate origin of *Bd* is still being debated. The earliest discovered case of chytridiomycosis was from an African archived specimen of *Xenopus laevis* from 1938. The next earliest case was from Canada in 1961. It is possible that the origin of *Bd* is in Africa, but more studies on archived specimens are needed. If Africa were the origin, greater genetic variation in *Bd* may be expected there than elsewhere. This is something that could be assessed to help elucidate *Bd*'s origins (Weldon et al., 2004).
References


Chapter 2: Lipid Biosynthesis During Zoosporangia Maturation

After mitotic divisions in a Bd thallus, the thallus becomes multinucleate. During subsequent zoospore production, these nuclei and cytoplasmic components must be divided and packaged into newly formed cellular membranes. This process must require vast lipid stores for membrane formation. After cytoplasmic contents have divided and new zoospores have been formed, multiple lipid bodies are present in each spore (Berger et al., 2005; Longcore et al., 1999). These lipid stores are likely used as energy sources in zoospores since Bd apparently does not obtain energy during this motile life stage (Piotrowski et al., 2004; Berger et al., 2005). These stores must then be assembled during the packaging of the newly forming zoospores.

The goal of the present study was to analyze lipid biosynthesis in zoosporangia using confocal microscopy to measure changes in lipid intensity as the sporangia mature. Given that lipids are necessary for the production of cellular membranes and would be required in motile zoospores as an energy source, it was expected that lipids would be synthesized and accumulate in the zoosporangia as they matured. At the time of zoospore production, lipid stores would then be divided among developing zoospores. These processes would result in increased lipid intensity signals until mitotic divisions and zoospore production, followed by the spread of lipid and the fluorescent signal over a larger area, causing lower intensity values. Because the zoosporangia used in this study mature and release zoospores after approximately 72 hours of development, the time points analyzed were 12, 24, 36, and 50 hours to capture zoosporangia as they mature but to end imaging before any zoospore release had started.
In this study, Nile Red was used as the lipid probe; the stain is a vital, fluorescent dye that allows for the detection of intracellular lipids. It has been used in flow cytofluorometry (Greenspan et al., 1985) and to image cytoplasmic lipid droplets using fluorescence microscopy (Goldstein et al., 1979). Nile Red is a selective dye in that the signal for the stain interacting with cytoplasmic lipid droplets can be isolated from the signal from cellular membranes using an excitation wavelength of 450-500nm and only collecting emission wavelengths less than 570 nm. Emission wavelengths greater than 590 nm are due to lipid signals from cellular membranes. Therefore, Nile Red represents a selective fluorescent hydrophobic dye for observing intracellular lipid. Furthermore, its fluorescence is quenched in aqueous environments, so fluorescence is only seen from the desired lipid environment (Greenspan et al., 1985). Nile Red is also often used in confocal microscopy due to its high photochemical stability (Basting et al., 1976).
Materials and Methods

**Bd Culture Maintenance**

*Bd* (strain JEL423) was kindly provided by Dr. Joyce Longcore (University of Maine). The fungus was cultured on 1% tryptone/1% agar plates. Every 3-4 days, plates were flooded with sterile deionized water (dH$_2$O) to collect released zoospores to be used to inoculate fresh plates.

**Growth on Coverslips**

Zoospores were collected from petri plates as described above and diluted in 1% liquid tryptone media to approximately $2 \times 10^6$ zoospores/mL. Five hundred microliters of this solution was added to the top of a cleaned, sterile coverslip. The coverslip was incubated at room temperature for the desired number of hours to allow zoospore adherence to the glass and maturation. This process was repeated to create 4 time points of interest (12, 24, 36, and 50 hrs) for lipid staining such that all coverslips reached the appropriate incubation length at the same time and could undergo simultaneous staining from a master mix to control for any staining procedure variability.

**Staining Procedure**

After incubation of the coverslips, they were washed once with dH$_2$O before a 5 min fix in a 2% formaldehyde solution. The coverslips were then washed 3 times with 50 mM Tris-HCl (pH 7.5). A solution of 1.4µg/mL Nile Red (Sigma Life Science, St. Louis, Missouri) and 0.1% dimethyl sulfoxide (DMSO) in tryptone was added to each coverslip and incubated at room temperature for 10 minutes. During this incubation, and through all subsequent microscopy, coverslips were kept shielded from light in order to limit
photobleaching. Coverslips were then added to cleaned slides and sealed at the edges with clear nail polish.

**Confocal Procedure**

Imaging was performed using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, LCC, Thornwood, New York) and a 40x Plan-Apochromat lens (0.95 NA). The pinhole was maintained at 1 airy unit to maximize resolution. ZEN software (Carl Zeiss) was used for image acquisition. Excitation of intracellular Nile Red was performed using a 488 nm Argon laser. Laser power and detector settings were held constant across all samples. The emission range captured was 539-632 nm to collect signals from both cytoplasmic lipids and those associated with cellular membranes. Transmitted light images were taken simultaneously with fluorescent scans. Ten independent fields of view were recorded per time point. To capture all available data regarding lipid content in zoosporangia, z-stacks with 0.96 µm step intervals were collected. Pixel averaging of 4 was used to reduce noise acquisition and bi-directional scanning was performed at high pixel density (1024x1024 pixels).

**Image Processing and Analysis**

ZEN software was used to create a maximum intensity projection for each field of view. The analyze particles tool in Image J (National Institutes of Health, Bethesda, Maryland) was used to calculate mean and maximum lipid intensity following thresholding. Maximum intensity measurements and an over-under lookup table were used to confirm that no information was lost due to intensity peak clipping. For particle analysis, threshold values were held constant across different fields of view within each time point. Each time point was assigned an independent threshold value to account for
varying intensities and to keep equivalent size of the lipid signals measured. Intensity values were given using an 8-bit intensity scale of 0-255 arbitrary units. R software was used to conduct ANOVA and Tukey HSD post-hoc tests between mean signal intensities and mean number of lipid droplets per sporangium within each trial. Adobe Photoshop and Adobe Illustrator (Adobe Systems, San Jose, California) were used to resize the images and assemble figures.
Results

In order to analyze lipid biosynthesis in zoosporangia and how lipid content relates to zoospore production, mean lipid intensity values were measured from fixed sporangia at 4 different time points. It was expected that lipid intensity would increase as sporangia matured, owing to lipid biosynthesis in preparation for zoospore production. During zoospore production, lipid intensity values were expected to decrease as lipids were spread out from storage and packaged into new cellular membranes and energy stores for developing zoospores and were thus present in less dense accumulations. The lipid biosynthesis experiment was independently performed 3 times. Results from the first trial were consistent with initial expectations. The mean lipid intensity values for 12, 24, 36, and 50 hours were 67.95, 84.59, 87.34, and 72.02 8-bit intensity units, respectively (Table 1). These data are consistent with the hypothesis that an increase in intracellular lipid would occur as sporangia prepare to assemble new zoospores. The decrease in lipid intensity at about 50 hrs was also expected as lipid stores would be divided among newly forming zoospores around this time.

The second and third trials were inconsistent with the first. In trial 2, very few zoosporangia were adhered to the 12 hour coverslip after the washing and staining steps. It is possible that the washing steps administered to that specific treatment were performed more harshly, resulting in disturbance of sporangial adherence. Lipids spots from only 3 zoosporangia were available for measurement at the 12 hour treatment, producing a mean lipid intensity of 82.79. However, the small sample size likely renders this number unreliable. The 24, 36, and 50 hour treatments had mean lipid intensities of 52.02, 79.22, and 57.37, respectively. It is possible that these data could fit the expected
trend described above had there been a more accurate measurement of the 12 hour treatment since intensity did increase between 24 and 36 hours and then fell by 50 hours. However, the lipid intensity for the 24 hour treatment was lower than was expected.

The third trial (images in Figure 1) also showed a lower mean intensity for the 24 hour treatment than was expected. The intensity means for 12, 24, 36, and 50 hours were 77.29, 61.72, 81.84, and 83.11, respectively. The third trial did not show the same drop in intensity at 50 hours that was seen in trials 1 and 2.

ANOVA and Tukey post-hoc analyses revealed significant differences (p < 0.001) in mean intensity values for all treatments within a trial except for between the 12 and 36 hour samples from Trial 2, which were not significantly different from each other (p=0.94)

It was expected that the number of lipid accumulations (fluorescent aggregations of lipid, hereafter referred to as “lipid droplets”) present in zoosporangia would increase as they matured due to both ongoing lipid biosynthesis and the dispersal of lipid stores when packaging lipids into cellular membranes for zoospore production. As expected, greater numbers of lipid droplets were present and analyzed per sporangium as zoosporangia matured (Table 1). Across all trials, coverslips incubated for 12 hours averaged the fewest lipid droplets present per sporangium (1.65, 3, and 1.67 droplets, respectively). As mentioned above, the coverslip from trial 2 had very few zoosporangia adhered, owing to only 1 sporangium with 3 lipid droplets being analyzed. Twenty-four hour coverslips averaged 2.83, 3.47, and 2.34 lipid droplets per sporangium, respectively. Thirty-six hour coverslips averaged 5.71, 5.32, and 7.70 droplets, respectively. Fifty-
hour coverslips averaged 4.94, 9.70, and 6.39 droplets, respectively. The increasing numbers of intracellular lipid accumulations as zoosporangia matured was seen in all trials. All averages were significantly different from each other in trial 1, the 50 hour sample was significantly different from all other samples in trial 2, and all samples were significantly different in trial 3 ($p < 0.05$) except the difference in lipid droplet number per sporangia between 12 and 24 hour samples, which were not significantly different.
Discussion

While results were inconsistent, a possible trend was seen in increasing lipid intensity prior to the 50 hour adherence mark. Further experimentation is needed to investigate this trend. If indicative of lipid activity in zoosporangia \textit{in vivo}, the data is suggestive that lipids are synthesized and accumulate over time in zoosporangia as they mature. Once the nuclei in sporangia divide and new zoospores start to form, lipids are allocated to each developing spore for use in creating cellular membranes and energy stores, which is supported by the decrease in intensity of lipid signal observed in the 50 hr sporangia in trials 1 and 2. It is possible that lipid accumulation is temporally variable enough that the drop in lipid intensity would have occurred later in the life cycle of the sporangia in trial 3 and that these data would have supported the trend seen in trials 1 and 2 if given a later imaging time point. All mean lipid intensity values were significantly different within each trial except for in trial 2 when comparing the 12 hour sample to the 36 hour sample, likely due to the low sample size in the 12 hour treatment.

Further supporting lipid synthesis and accumulation over time in maturing zoosporangia are the increases in numbers of lipid droplets per sporangium over time until the 36 hour post-inoculation mark. The increased number of lipid droplets measured is due to the accumulation of lipids that are synthesized in preparation for zoospore production as the sporangia matures and may be more indicative of lipid biosynthesis than the intensity measures. The change in lipid number per sporangium from 36 hours to 50 hours was not a uni-directional change. It may be that lipid accumulation can be completed prior to the 50 hour mark.
The most accurate method of measuring lipid biosynthesis and intensity over time would be to use a time-lapse recording of 1 field of view of sporangia from adherence to maturation. An 8 hour time-lapse was attempted as the initial confocal procedure in this study. It was seen that, even with low laser power and minimized pixel-dwell time, moderate Nile Red photobleaching limited the possible length of the time lapse to one in which not much change could be seen. Thus, it was more advantageous to sacrifice paired sampling in order to measure a greater time range in the sporangia life stage. In the future, studies using alternative fluorescent lipid dyes should be employed to find a dye with high enough photostability to allow for 8+ hours of scan time without significant photobleaching.

In addition, lipid analysis throughout the spore stage should be conducted. Since zoospores rely exclusively on lipid stores for energy (Piotrowski et al., 2004; Berger et al., 2005), depletion of lipids and decreased mean intensity signals would be expected over time. Zoospores usually encyst by 24 hours of activity. Piotrowski et al. (2004) demonstrated that, after 24 hours, only 5% of zoospores were still motile. It is possible that encystment (in the absence of a host) occurs due to lipid stores reaching a critically low level. Furthered studies on the energetics of zoospores would enhance the understanding how Bd develops and matures, especially during saprophytic growth.
Table 1: Data from 3 confocal trials. Included are the average lipid intensity values gathered from maximum intensity projection images and the average number of lipid droplets present per sporangium for 12, 24, 36, and 50 hours treatments.
<table>
<thead>
<tr>
<th>Trial</th>
<th>Average Lipid Intensity</th>
<th>Average Number of Lipid Droplets per Sporangium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>Trial 1</td>
<td>67.95a</td>
<td>84.59b</td>
</tr>
<tr>
<td>Trial 2</td>
<td>82.79a</td>
<td>52.02b</td>
</tr>
<tr>
<td>Trial 3</td>
<td>77.29a</td>
<td>61.72b</td>
</tr>
</tbody>
</table>

Within each trial and measurement, different letters in superscripts indicates statistically significant differences (p<0.05).
Figure 1: Confocal imaging of intracellular lipids in *Bd* by Nile Red fluorescent dye.

Four time points in the sessile, zoosporangia stage of the life cycle were used to visualize lipid content changes as zoosporangia mature. Included are images from the red fluorescent channel and transmitted light images. (A) Images of zoosporangia adhered to the coverslip for 12 hours or less. (B) Images from zoosporangia adhered for 24 hours or less. (C) Images from zoosporangia adhered for 36 hours or less. (D) Images from zoosporangia adhered for 50 hours or less.
References


Chapter 3: Density-dependent Zoosporangia Maturation Rate and Potential Developmental Regulators of This Phenomenon

Life cycle duration, and thus maturation rate, depends on multiple factors, e.g., temperature, pH, and culture medium (Piotrowski et al., 2004). Through serial sub-culturing with an uncontrolled number of zoospores added to new plates, it was observed that more densely populated cultures would mature up to a day sooner than less densely populated ones. The length of the zoosporangia discharge tube is also affected by density of culture (Berger et al., 2005).

In order for zoosporangia to sense an environment of high sporangial density and increase their maturation rates, it was hypothesized that zoosporangia may be secreting factors that act as signals to surrounding zoosporangia to enhance development. More densely populated cultures would have more of this factor present in the surrounding media and would cohesively mature sooner than less densely populated cultures. Chemical signaling between conspecifics is commonly seen in nature as a way to synchronize reproduction (Koenig and Knops, 2005), warn against predators (Seyfarth et al., 1980), signal for mates (Rich and Hurst, 1998), or affect development of nearby organisms. Studies have also shown that some secondary metabolites secreted by the mycelia of other species of fungi can induce sexual or asexual sporulation in conspecifics (Park and Robinson, 1969; Hicks et al., 1997)

Quicker maturation would be advantageous if zoosporangia develop synchronously in the invasion and infection of a host. It is also possible that zoosporangia may detect excreted metabolites of nearby sporangia and increase their
maturation rate in order to more quickly release zoospores that can exploit the environment before resources are depleted.

The aim of this project was to demonstrate the density-dependent maturation rate of zoosporangia and to determine a potential molecular weight range for the putative secreted factors involved in mediating this quickened development. This was achieved by first testing whether or not cultures with higher zoospore concentrations matured faster than those with lower concentrations. Then, supernatant from zoosporangia cultures was collected to test if the presence of conditioned media, and any factors dissolved in it, would increase maturation rates and if a gradient of media concentrations would result in different maturation rates. A similar study using *Penicillium notatum* found that medium taken from a mature culture enhanced sporulation rate of mycelia placed in it compared to mycelia in fresh medium (Hadley and Harrold, 1958). Finally, to isolate developmental regulator candidates by determining a possible molecular weight range for the molecule responsible, conditioned media was frozen and fractionated into two different molecular weight ranges (less than 50 kDa and greater than 50 kDa) before use in a maturation assay.

After showing that culture density affects growth and that factors in the supernatant may be mediating this phenomenon, all assays were performed with only 16,000 zoospores per well to limit the effect that their natural secretion of any compounds of interest could enhance maturation in the wells.
Materials and Methods

Bd culture maintenance

*Bd* (strain JEL423) was kindly provided by Dr. Joyce Longcore (University of Maine). The fungus was cultured on 1% tryptone/1% agar plates. Every 3-4 days, plates were flooded with sterile dH$_2$O to collect released zoospores. This spore/water mixture was used to inoculate fresh plates.

Initial Density-Dependence Assay

Freshly-released zoospores were collected from a tryptone/agar plate as described above and diluted to $1 \times 10^6$ zoospores/mL with 1% tryptone. One mL of 1% tryptone was added to 10 wells of a 12-well plate. To two wells, 200,000 zoospores were added. Similarly, 100,000, 50,000, 25,000, and 12,500 zoospores were added to two wells each. The plate was placed on an orbital shaker for 60 seconds at 60 rpm to evenly distribute spores within the well and was then set aside for 72 hours to allow zoospores to adhere to the plastic and grow into zoosporangia. At 72 hours, and once every 6 hours until the 94 hours post-inoculation mark, mature zoosporangia were counted. The percentage of mature sporangia (with zoospores already released or in the process of release) out of total sporangia present was calculated for one field of view from each well. The mean percentages of mature zoosporangia for each replicate were calculated.

Supernatant Collection

To obtain supernatant containing all molecules potentially secreted by the zoosporangia, 200,000 zoospores in 1mL of 1% tryptone were added to each well of a 12-well plate. The plate content was mixed as described above. After 72 hours, prior to any zoospore release, the supernatant was collected and centrifuged at 3000 x g for 5
minutes to remove any zoospore or cellular debris. The supernatant was then frozen at -80°C in 200 µL aliquots for storage.

**Supernatant Assay**

To test the effect of the supernatant presence on maturation rate, a 12-well plate was created with 6 control wells of 16,000 zoospores in 1mL of 1% tryptone and 6 wells of 16,000 zoospores in 800 µL of 1% tryptone and 200 µL of supernatant. This plate was then mixed as described above. Approximately 75-79 hours later, when sporangia were starting to mature and release zoospores, 4 fields of view from each well were microscopically examined. The percentage of mature sporangia was calculated for each field of view. These percentages were then averaged for all fields from control and treated wells. The count was repeated every 6 hours until all sporangia were empty or until newly adhering zoospores began obstructing counting.

**Differing Supernatant Concentration**

To test whether a range of supernatant concentrations would result in a matching range of zoosporangia maturation rates, a 12-well plate was set up with 5 treatments (2 replicates per treatment). To the first two wells, 16,000 zoospores were added to 950 µL of 1% tryptone and 50 µL of supernatant. The second two wells consisted of the zoospores, 900 µL of 1% tryptone, and 100 µL of supernatant. The 3rd, 4th, and 5th sets of wells were set up in the same fashion with 150, 200, and 250 µL of supernatant added, respectively. Counts were performed every 5-6 hrs starting at 85 hours post-inoculation.

To expand upon the results from the assay above, a 12 well plate was prepared with 4 control wells possessing 16,000 zoospores, 1 mL of 1% tryptone, and no supernatant, 4 wells of zoospores treated with 200 µL supernatant and 800 µL tryptone,
and 4 wells of zoospores with 250 µL supernatant and 750 µL tryptone. Percentages of mature sporangia per treatment were determined as above. The numbers of mature sporangia were recorded at 6 hour intervals.

**Fractionating the Supernatant**

Frozen supernatant, which had previously been subjected to centrifugation at 3000 x g, was thawed, passed through a 0.45 µm filter, and then passed through a 0.2 µm filter to remove debris. Three mL of the supernatant were then subjected to a one-step fractionation using a 50,000 Da MW filter (Pall Life Sciences, Port Washington, New York) by centrifugation using a fixed-angle rotor in a clinical centrifuge for 40 minutes. The eluate (molecules <50 kDa) and the retentate (molecules >50 kDa) were collected. The retentate was then resuspended in 1 mL of dH₂O by vortexing and repeated pipetting. The resuspended retentate was subjected to fractionation again to ensure that all molecules of less than 50 kDa were removed. Finally, a volume of dH₂O equal to the amount of supernatant initially passed through the filter was added to the upper chamber of the filtration device, and the retained molecules were resuspended. This fraction was stored at -80°C as the supernatant fraction containing molecules greater than 50kDA.

**Fractionation Assay**

The maturation rate assay using 16,000 zoospores per well was repeated with 6 control wells, 6 wells with 200 µL of unfractionated supernatant, 6 wells with 200 µL of the supernatant fraction containing less than 50 kDa molecules, and 6 wells with 200 µL of supernatant fraction containing greater than 50 kDa molecules. Mature sporangia were counted approximately every 6-7 hours after 76 hours post-inoculation.
Statistical Analysis

R software was used to conduct one-way ANOVA and Tukey post-hoc tests to analyze differences in the percentage of sporangia that were mature within each time point post-inoculation. Homogeneity of regression slopes tests were used to measure if the rate of maturation differed among treatments within each assay.
Results

Initial Density-Dependence Assay

At 72 hours post-inoculation, clearly disparate counts of mature sporangia were observed across the different treatments (Figure 1). The treatment with only 6,500 zoospores/well showed zero mature sporangia, whereas the densest treatment (200,000 zoospores/well) showed approximately 52.7% mature sporangia by 72 hours post-infection. The number of mature sporangia in wells treated with 200,000 zoospores was significantly higher than the number from any other treatment at both 72 and 78 hours post-inoculation (p < 0.05). The wells with 6,500 zoospores added had the lowest percent of mature sporangia of all wells until the 94 hr post-inoculation mark, and this percentage was significantly lower than that of every other treatment at the 84 hr post-inoculation mark (p < 0.05). By 94 hr, the least densely populated wells still averaged over 23% fewer mature zoosporangia than the most densely populated wells. As expected, the percent of mature sporangia within a time point tended to increase with an increased number of zoosporangia present. While there was some overlap in the percent of mature sporangia seen in the wells with 12,500, 25,000, and 50,000 zoospores added, they were always lower than the rates for the wells with 100,000 zoospores and above the rates for those with 6,500 zoospores. It is clear from these data that there is a density-dependent effect on the maturation rate of *Bd*. The homogeneity of regression slopes showed no significant differences in maturation rate among treatments.

Supernatant Assay

In the assay comparing control wells with no supernatant added to treatment wells with supernatant added, there is a clear increase in the percent of mature sporangia in
treated wells (Figure 2), and this increase is significant (p < 0.001) at every time point post-inoculation except at 99 hours. At 75 hours post-inoculation, control wells revealed approximately 2.8% maturation, while treated wells showed 13.2% maturation. By 87 hours post-inoculation, control wells had reached 22.5% maturation, while treated wells exhibited 57.5% maturation. The increased numbers of mature sporangia in treated wells continued until the 99 hr post-inoculation mark where most sporangia (approx. 90%) had already matured in both control and treated wells. The homogeneity of regression slopes showed no significant difference between the maturation rates of both samples.

**Differing Supernatant Concentration**

From the two maturation assays using conditioned media and described above, an experiment examining if a concentration-dependent effect of the conditioned medium existed, it was found that higher concentrations of supernatant resulted in earlier maturation of zoosporangia than was seen with lower concentrations (Figure 3A, B). Throughout the duration of the experiment with 5 treatments, wells that had 250 µL of supernatant had a consistently higher percent of zoosporangia mature than all other wells. In addition, the wells that were treated with 50 µL of supernatant had the lowest percent of mature sporangia at every time point after 85 hours post-inoculation. The second most concentrated supernatant treatment (200 µL) showed percentages of zoosporangia maturation consistently just lower than the 250 µL treatment. At the 97 hour post-inoculation point, the number of mature sporangia was significantly different when comparing wells with 250 and 50 µL of conditioned media (p < 0.01) and when comparing wells with 200 and 50 µL of conditioned media (p < 0.05).
In the assay using only 3 treatments (control, 200 µL supernatant, and 250 µL supernatant), the results show that both supernatant-treated wells exhibited earlier maturation of sporangia. The wells with 200 µL of supernatant unexpectedly showed quicker maturation than the wells with 250 µL of supernatant, but they both revealed an earlier maturation than control wells at every time point prior to 105 hours post-inoculation. At 92 hours post-inoculation, the number of mature sporangia in wells treated with 200 µL of conditioned media is significantly higher than that of the control wells (p < 0.01). At 99 hours post-inoculation, the numbers of mature sporangia from both treated wells are significantly higher than that of the control wells (p < 0.001).

The homogeneity of regression slopes analyses for both assays showed no significant differences in maturation rates between treatments.

**Fractionation Assay**

The addition of unfractionated supernatant resulted in the earliest maturation (Figure 4). Wells with either greater than, or less than, 50KDa fractions resulted in very similar maturation that was later than that of wells with unfractionated media but earlier than that of control wells. At 83 hours post-inoculation, unfractionated wells had significantly higher numbers of mature sporangia than control wells (p < 0.05). At 90 hours post-inoculation, all 3 treated wells had significantly greater numbers of mature sporangia than the control wells (p < 0.005). At 96 hours post-inoculation, wells with unfractionated media and wells with less than 50 kDa fraction added had significantly greater numbers of mature sporangia than control wells (p < 0.05). No significant differences were seen in the number of mature sporangia at the 102 hour post-inoculation mark. The homogeneity of regression slopes showed a significant difference between the
maturation rate of the control wells and the wells with the addition of media containing molecules greater than 50 kDa (p = 0.002).
Discussion

The results from the density-dependent growth assay support the notion that culture density affects how early zoosporangia mature. The results of both the supernatant addition assay and the assays involving a concentration gradient of supernatant added support that increased supernatant concentrations cause earlier maturation of zoosporangia. These findings suggest that a molecule (or molecules) is secreted from zoosporangia that aids in shortening the time until maturation of nearby sporangia. In addition, more conditioned media present means more of the agents in question are present, resulting in even earlier maturation.

In the fractionation assay, both media fractions resulted in later maturation than the unfractionated data but earlier maturation than the control data. This supports that there may be multiple agents of differing molecular weights that are secreted from zoosporangia and aid in quickening maturation of nearby sporangia and that they may work in a synergistic fashion, which may explain the earlier maturation seen from unfractionated supernatant addition. Because wells containing the greater than 50 kDa fraction resulted in a different maturation rate than the control wells, the study merits further investigation. Because wells with both less than and greater than 50 kDa conditioned media fractions exhibited earlier maturation than control wells, this study should be continued by fractionating the media further. Using a 30 kDa and 80 kDa filter, for example, in addition to the 2 used in this study would create 4 media fractions that may help identify the molecular weights of these agents. The fractions could then be run on a gel using SDS-PAGE to see what proteins are present. Proteins determined to be
possible developmental regulators could then be purified and used in proof-of-function maturation assays.

That *Bd* can regulate and quicken the growth of nearby conspecifics may contribute to how quickly infection by *Bd* can overwhelm its host. Within a couple weeks of infection, susceptible hosts begin to die. In addition to aiding more rapid host invasion, secreting molecules that cause earlier maturation could perhaps aid in competition against bacteria that invade the lesions created by *Bd* infection.
Figure 1: The effect of culture density on zoosporangia maturation rate. Density-dependent maturation assay of 6 different inoculation numbers of zoospores.

Figure 2: The effect of the addition of conditioned media on zoosporangia maturation rate. Maturation assays of 16,000 zoospores per well with treatment group receiving 200 µL of zoosporangia supernatant.
Fig 1.

Fig 2.
Figure 3: The effect of differing supernatant concentrations on zoosporangia maturation. 

(A) Maturation assay utilizing 5 different supernatant addition amounts to wells with 16,000 zoospores. (B) Maturation assay comparing control wells with 16,000 zoospores and no supernatant addition to wells with 16,000 zoospores and either 200 or 250 µL supernatant.
Fig. 3 (A&B)
Figure 4: The effect of different supernatant fractions on zoosporangia maturation.
Maturation assay after supernatant fractionation into greater than and less than 50 KDa molecular weight fractions. Maturation rates from control wells without supernatant are compared to rates from wells with unfractionated supernatant, supernatant containing less than 50KDa molecules, and supernatant containing greater than 50KDa molecules.
Fig. 4

![Graph showing percent mature sporangia over hours post-inoculation for different samples: control, unfractionated, <50K, and >50K.](image-url)
References


Appendix: Isolation of Pathogenicity Factors

One of the most important and unresolved questions regarding Bd is how the fungus first colonizes and then disrupts epidermal structure and machinery. Bd may release digestive enzymes or a toxin that aid in penetration of the skin, disruption of skin function and electrolyte transport, and subsequent pathogenesis, and this molecule or compound could be one that also affords immune evasion capabilities (Voyles et al., 2011). Bd’s genome shows metalloprotease, aspartyl-protease, and serine-type protease expansions; these proteases are expressed by many pathogenic fungi and may be used for host substrate or peptide degradation (Monod et al., 2002; Rosenblum et al., 2008; Joneson et al., 2011). In addition to the mentioned proteases, many other molecules might be involved in host invasion by Bd, but the mechanism for this process is still unknown. It is crucial that this mechanism be elucidated as it will provide a greater understanding of the infection process in chytridiomycosis and could lead to the development of compounds that may inhibit or block this process.

The aim of this work was to analyze proteins secreted by or upregulated in Bd during the 2 different life stages to isolate potential protein candidates for the fungus’s invasion mechanism. Multiple extraction methods were employed in an attempt to discover a method that resulted in the clear bands for band excision from an SDS-PAGE gel. Candidate bands would have been sequenced and analyzed against known pathogenicity factors from other fungi. Trichloroacetic acid (TCA) is one of the most popular agents used in protein precipitation, even though multiple mechanisms have been proposed regarding the underlying mechanism behind TCA-induced precipitation (Dakshinamurthy et al., 2009). Multiple TCA extraction protocols were used in this
study in an attempt to maximum protein yield. As an alternative, an alkali method for protein precipitation was also conducted. Chloroform-methanol washes were used to remove lipids in an attempt to clear band smearing. To visualize protein bands, Coomassie staining and silver nitrate staining were used.
Materials and Methods

Zoospore Collection

Zoospores were collected by flooding a plate of mature zoosporangia with sterile dH₂O and pipetting the suspension into microcentrifuge tubes. The tubes were then spun at 10,000 x g for 5 minutes to pellet the zoospores. The supernatant was removed, and approximately a 1:1 ratio of Cellytic Y (Sigma Life Sciences) with 10 mM dithiothreitol and 15 µL of yeast protease inhibitor cocktail (Sigma Life Sciences) was added to the zoospore pellet and used to resuspend it. Tubes were then frozen at -80°C and were subjected to 3 cycles of freeze-thawing to form ice crystals in the cells to aid further lysis. Freeze-thawing was performed by bringing the temperature of frozen tubes quickly to 37°C using a hot water bath and then refreezing at -80°C. Later zoospore collections, as indicated, omitted the lytic cocktail and were performed using sterile dH₂O.

Sporangia Collection

Zoospores were collected from mature sporangia in sterile dH₂O as described above. A hemocytometer was then used to find the concentration of zoospores in the suspension. To sterile, empty petri plates (100 x 15 mm; Fisher Scientific, Hampton, New Hampshire), 1% tryptone media was added until the bottom of the plate was covered. Fifty million zoospores were added to each plate, and the plates were mixed at 60 rpm for 1 minute on an orbital shaker to evenly distribute zoospores. Plates were then incubated at room temperature for 24 hours. During this time, zoospores settled to the bottom of the plates, adhered to the plastic, and developed into germlings. After incubation, the liquid media was decanted from the plates, and the plates were placed into a -20°C freezer overnight. When plates were removed from the freezer, 6 drops of the
lytic cocktail described above were added to each plate, and the plates were scraped using a rubber policeman to collect the sporangia that had adhered. The liquid was transferred to microcentrifuge tubes, frozen at -80°C, and subjected to three cycles of freeze-thawing to aid cell lysis. Later sporangia collections omitted the lytic cocktail and were performed using sterile dH₂O.

**Initial TCA Extraction Method**

Three hundred microliters of zoospore and sporangia samples were placed in microcentrifuge tubes for protein extraction. Four hundred µL of a chloroform/methanol solution (2:1 v/v) was added to each tube and mixed by vortexing. Four hundred µL of 10% trichloroacetic acid (TCA) in acetone was added and vortexed. The tubes were placed on ice for 30 minutes to allow for protein precipitation. Tubes were then centrifuged at 10,000 x g for 5 minutes and the resultant aqueous layer was discarded. Tubes were vortexed again and washed with 600 µL of cold 10% TCA in dH₂O. The tubes were spun again, the aqueous layer was discarded, and the 10% TCA in dH₂O wash and the spin was repeated. Tubes were then washed, spun, and the supernatant discarded twice more with 1 mL of dH₂O, first, and 1 mL of cold acetone, second. Finally, tubes were washed and spun three times with 1 mL of 80% acetone.

**Modified TCA Extraction Method**

Three hundred µL of zoospore and sporangia samples were placed in microcentrifuge tubes for protein extraction. Three hundred µL of a chloroform/methanol solution (2:1 v/v) was added to each tube and mixed by vortexing. The chloroform/methanol step was then repeated. The procedure then followed the steps
listed above except that, in the very last step, only 1 wash with 80% acetone was performed.

Third TCA Extraction Method

To 4 volumes of sporangia and zoospore collections, 1 volume of 100% TCA was added. The tubes were incubated on ice for 10 minutes and then spun at 14,000 x g for 5 minutes at 4°C. The supernatant was removed and the protein pellets were resuspended in 300 µL of acetone. The spinning and acetone wash was repeated 2 more times. The protein pellet was then air dried at room temperature for 30 minutes or until the acetone had evaporated.

Fourth TCA Extraction Method

To 300 µL of zoospores and zoosporangia samples on ice, 300 µL of a TCA extraction buffer (10 mM Tris-HCl at pH 8, 25 mM NH₄Ac, 1 mM Na₂EDTA at pH 8, and 10% TCA) was added. The tubes were vortexed 5 times, for 1 minute each, and rested on ice between vortexing. The tubes were then centrifuged for 10 minutes at 16,000 x g to pellet the precipitated proteins along with cellular debris. After centrifugation, the supernatant was removed, and the protein pellet was resuspended in 150 µL of resuspension buffer (0.1M Tris-HCl at pH 11 and 3% SDS). Samples were then boiled for 5 minutes, cooled to room temperature, and centrifuged again for 30 seconds at 16,000 x g to pellet cell debris. The supernatants were then removed and transferred to new microcentrifuge tubes.

Modified Yeast Technique (Alkali)

This extraction procedure is an adaptation of work on yeast protein extractions by Kushnirov (2000). Approximately 100 x 10⁶ zoospores and sporangia were pelleted and
resuspended in 200 µL of a lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, and 2% β-mercaptoethanol). Tubes were incubated for 10 minutes at 90°C. Five µL of 4 M acetic acid was added to neutralize the pH and the tubes were vortexed for 30 seconds. Tubes were again incubated for 10 minutes at 90°C.

**Sample Buffer Only**

Zoospore and zoosporangia samples were collected in sterile dH₂O and frozen at -80°C. Immediately after the described freeze-thaw cycles, samples were mixed with 2X sample buffer, and prepared for electrophoresis as described below.

**SDS-PAGE Preparation**

One volume of 2X SDS sample buffer was added to each protein sample. Samples were boiled for 10 minutes prior to loading into a polyacrylamide gel. A 10% acrylamide gel was used to run all samples.

**Gel Processing**

Once the gels were finished running, they were removed from the electrophoresis apparatus and fixed in a 40% methanol/10% acetic acid fixative solution for 1 hour. Coomassie blue was used to stain the gel for 20 minutes. Then, the gels were slowly destained with a 20% methanol/10% acetic acid solution until band separations were clear. Gels were stored in a 5% acetic acid solution.

**Silver-Staining Detection**

An SDS-PAGE gel was loaded and run normally and then fixed for 1 hour in a solution of 50% methanol, 10% acetic acid, and 50 µL glutaraldehyde in 100 mL of dH₂O. After fixation, the gel was washed twice in 50% ethanol for 20 minutes per wash. The gel was then treated for 1 minute with a hypo solution (20 mg/mL of sodium
thiosulfate in dH₂O). Next, the gel was washed with dH₂O 3 times for 20 seconds each. After washing, the gel was treated with 200 mg/100 mL silver nitrate in dH₂O for 30 minutes. Again the gel was washed 3 times (20 seconds each) in dH₂O before being developed in 100 mL of the developing solution (6 g sodium carbonate, 2 mL of the hypo solution, and 50 µL glutaraldehyde in dH₂O). Development was stopped after 30 minutes with 5% acetic acid.
Results

Coomassie staining of SDS-PAGE gels resulted in hundreds of protein bands for each protocol used. When using the first TCA extraction method, gel lanes showed heavy smear due to lipid presence. The modified TCA extraction used a second chloroform/methanol wash to remove these lipids, but bands still were not sharp. TCA extraction methods 3 and 4 and the alkali modified yeast technique yielded no improvement in obtaining sharp bands. No protein bands developed when using the silver-staining protocol. Zoospore protein samples yielded clearer images than 24 hour samples, but both were needed to do a band comparison to analyze differences in protein expression.
Discussion

Due to inadequate protein concentration and separation and lipid presence in gels, analysis of potential protein candidates involved in *Bd* pathogenicity was not completed. In addition to the band separation issues and due to thousands of generations of subculturing and not being exposed to a host, it is possible that the *Bd* cultures used were no longer expressing the genes specific to invasion mechanisms. As an example, *Bd* on tryptone media does not form invasion structures like the germination tubes that are used during amphibian skin invasion. Instead, only small papillae are formed to release zoospores. More efficient protein extraction techniques are necessary to obtain adequate protein isolation before considering whether the lack of a host would affect invasion gene expression.

Possible next steps include using microconcentrators to concentrate and fractionate samples. In addition, protein extraction from the agar media itself should be attempted. Since the target proteins are expected to be secreted, the fungus itself may be unnecessary to include in the extraction. Zoosporangia could be grown on and then scraped off tryptone/agar plates prior to maturation. The agar could then be melted and homogenized. Target proteins could be isolated from agar using filtration and then mixed and boiled with sample buffer as described for use in SDS-PAGE.

Although this experiment was unsuccessful, efforts such as this must continue until the mechanism behind host penetration and invasion by *Bd* is known. Until this is elucidated, scientists cannot hope to directly target pathogenicity factors of the fungus as a way to prevent or treat infection and chytridiomycosis.


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