STUDIES OF *BEAUVERIA BASSIANA* PATHOGENICITY, SURFACE CHARACTERISTICS AND HYDROPHOBINS

By

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
PDT	Potato Dextrose agar supplemented with 5 µg/ml trimethoprim
SAB	Sabouraud Dextrose
SDY	Sabouraud Dextrose with Yeast Extract
TFA	Triflouroacetic Acid
WCA	Water Contact Angle
RFU	Relative Fluorescence Units
DTT	Dithiothreitol
IPTG	Isopropyl-thio-β-D-galactoside
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
LB	Luria Bertani broth

Abstract of Dissertation Presented to the Graduate School
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STUDIES OF *BEAUVERIA BASSIANA'S* PATHOGENICITY, SURFACE CHARACTERISTICS AND HYDROPHOBINS

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The entomopathogenic fungus, *Beauveria bassiana* represents a promising biological control agent for insects and other arthropods, and is increasingly being studied as a model organism for examining fungal development and pathogenesis. Fungal host-pathogen interactions were examined by studying *B. bassiana* virulence towards a range of human and animal relevant tick species including *Dermacentor variabilis*, *Ixodes scapularis*, *Rhipicephalus sanguineus*, *Amblyomma americanum*, and *Amblyomma maculatum*. Fungal development and pathogenesis was studied via elucidation of the surface characteristics of the *B. bassiana* conidial spore, the major dispersal and infectious propagule produced by the fungus.

Ticks are considered major vectors of animal and human diseases second only to mosquitoes. The effective reduction and control of tick populations remains difficult, and the ability of *B. bassiana* to infect a range of tick species was examined. Adult and nymphal ticks were treated with different *B. bassiana* cell phenotypes. Dose-dependent mortality toward *Dermacentor variabilis, Rhipicephalus sanguineus*, and *Ixodes scapularis*, the latter the major disease vector for the Lyme disease causing spirochete, was determined. These data demonstrated that *B. bassiana* could be effective in

targeting ticks. A differential susceptibility towards certain tick species e.g. *A. maculatum* and *A. americanum* was noted with the former very susceptible and the latter more resistant to fungal infection. Results indicated that inoculum conditions can greatly affect successful virulence and subsequent mortality towards ticks.

Treatment of ticks with fungal cells and their cell free culture supernatant resulted in increased mortality. HPLC analysis of the spent growth media revealed oxalic acid as a major metabolite secreted by *B. bassiana* during growth suggesting that oxalic acid may contribute to virulence in *B. bassiana*. This hypothesis was supported by experiments which suggest that oxalic acid displays a pH dependent toxicity towards ticks, indicating that its secretion by the fungus during infection of target hosts plays a role in virulence.

Cell surface attachment is the first step in establishing mycosis and studies examining the attachment properties of the different *B. bassiana* cell types revealed that aerial conidia are able to adhere rapidly to both hydrophobic and hydrophilic surfaces. Cell surface hydrophobicity, adhesion, and spore dispersal are partly attributed to a proteinacious sporecoat called the rodlet layer, presumably consisting of proteins known as hydrophobins. Hydrophobins are small amphipathic proteins involved in the formation of aerial structures, attachment of fungal cells to surfaces, and self-assemble into characteristics 2-dimensional arrays.

The *B. bassiana hyd2* gene which codes for the Hyd2 hydrophobin was expressed in *Escherichia coli* as a fusion protein in partner with the Ssp DnaB intein domain derived from the *Synechocystis sp* DnaB intein. The protein was purified from inclusion bodies and reconstituted in an active form. Self-assembly of the purified

protein was monitored via microscopy (AFM and SEM), an amyloid assembly assay based upon Thioflavin T binding, and by contact angle measurements. In addition, the purified protein was used in trans-complementation assays of a *B. bassiana hyd2* targeted gene knockout.

CHAPTER 1
PATHOGENESIS OF *Beauveria bassiana* TOWARDS TICKS

Introduction

Infection by *B. bassiana* is a result of direct penetration of insect cuticle. This penetration uses a combination of chemical, enzymatic and mechanical methods that allows for a wide array of host susceptibility. Strains of *B. bassiana* have been shown to be pathogenic towards hard and soft ticks, especially members of the Ixodidae and Argasidae family of ticks and we postulate that it is a promising method for their control (Benjamin *et al.*, 2002; Kirkland *et al.*, 2004b). In order to better understand the efficacy of *B. bassiana* as a biocontrol agent towards ticks, an investigation into the virulence towards hard tick species *Dermacentor variabilis, Ixodes scapularis*, *Rhipicephalus sanguineus, Amblyomma americanum* and *Amblyomma maculatum* has been made. Our objective was to investigate the virulence of *B. bassiana* towards these important disease carrying tick species. The hypothesis was that the entomopathogenic fungi *B. bassiana* can be used as an effective means for the reduction and control of tick populations.

To further elucidate the specific mode of action during host pathogen interactions between *B. bassiana* and tick species we have determined that a secondary metabolite called oxalic acid is secreted and plays a role in its diverse host pathogenicity. Metabolic acids have been shown to mediate virulence towards some species of grasshopper (Bidochka & Khachatourians, 1991). High concentrations of oxalic acid in plants are thought to discourage insect foraging and have been shown to be toxic to honey bees and other plant pests (Alverson, 2003; Franceschi & Horner, 1980; Gregorc & Poklukar, 2003; Horner & Zindlerfrank, 1980; McConn & Nakata, 2002; Nakata,

2002). Treatment of ticks with fungal cells and their cell free culture supernatants resulted in >50% mortality within 14 days as compared to almost no mortatality using deionized H_20 or fresh growth media. This would indicate the presence of some important virulence factors secreted into the spent media. HPLC analysis of the spent growth media revealed oxalic acid as a major metabolite. My hypothesis was that oxalic acid is an entomopathogenic virulence factor of *B. bassiana*. My objective was to investigate the acaracidal activity of cell-free fungal culture supernatants. The results suggested that oxalic acid displays a pH dependent toxicity towards ticks and that its secretion may help account virulence against insects (Kirkland *et al.*, 2005).

Literature Review

General Biology of *Beauveria bassiana*

Beauveria bassiana is a filamentous fungus of the Deuteromycete (Ascomycota) in the order of Hypocreales. It is a haploid organism with eight chromosomes and a genome size of 34-44Mb (Viaud *et al.*, 1996). Named after Augistino Bassi in the 1830's it was discovered initially infecting silkworms. It is an opportunistic entomopathogen and endophytic organism. *B. bassiana* is found on the surface of insects as white to yellowish conidiospores (Fig 1-1). The life cycle is most often biotrophic beginning with attached conidiospore penetrating, colonizing, exploiting, and finally producing progeny conidiospores for dispersal onto other susceptible hosts. It is under study as a biological control agent due to its broad entomopathogenic host range (Clarkson & Charnley, 1996; Ferron, 1981; Kaaya & Munyinyi, 1995; Klinger *et al.*, 2006; Kucera & Samsinak.A, 1968; Leathers *et al.*, 1993; Maurer *et al.*, 1997; McCoy, 1990; Reithinger *et al.*, 1997) and contains a sporecoat protein composed of hydrophobins (Bidochka *et al.*, 1995b; Holder & Keyhani, 2005). *B. bassiana* produces

three mononucleated cell types; aerial conidia, blastospores, and submerged conidia (Bidochka *et al.*, 1987; Thomas *et al.*, 1987). Aerial conidia are 4-5µm in size with a round or oval shape and contain an outer rodlet layer. They are produced on solid nutrient substrates such as insect and plant hosts, or nutrient agar. Blastospores are 6-12µm in size and have a hot dog shape and do not contain a rodlet layer. These spores are produced in nutrient liquids such as sabouraud dextrose media. Submerged conidia are the smallest of the spores ranging from 2-4 µm in size with a round to oblong shape and also do not contain a rodlet layer (Holder & Keyhani, 2005). Each cell type has unique surface binding properties allowing for differential attachment to substrata (Holder & Keyhani, 2005; Leland *et al.*, 2005).

Biocontrol of Ticks

Biological control is the use of natural enemies towards an invasive host target. As an alternative to harsh chemical treatments, *B. bassiana* is widely used as an addition to integrated pest management strategies due to its broad host range. Because *B. bassiana* is considered to be non-pathogenic to humans it is a useful target for the study of alternative pest control that is both commercially viable and environmentally friendly. However, studies have shown *B. bassiana* carries reactive allergens, but the few cases of human infection by *B. bassiana* have been seen in individuals who are immunocompromised (Henke *et al.*, 2002; Kisla *et al.*, 2000; Westwood *et al.*, 2005).

Currently, ticks are considered one of the major vectors of human infectious diseases second only to mosquitoes in their ability to transmit diseases (Parola & Didier, 2001). They are also a major concern for livestock animals in specific areas (Polar *et al.*, 2008). Ticks are obligate hematophagous arthropods that parasitize almost every class of vertebrates. Lyme disease, babesiosis, tick-borne encephalitis, granulocytic

ehrlichiosis, tick bite fever (Rocky Mountain Spotted fever), and tularemia are transmitted when the tick engorges on a blood meal from an animal host (Coyle, 2002; Keirans *et al.*, 1996; Mavtchoutko *et al.*, 2000; Parola & Didier, 2001; Piesman *et al.*, 1999; Singh-Behl *et al.*, 2003; Walker, 1998). Chemical acaracides such as organophosphates, carbamates, and pyrethroids are often used for successful reduction and control of tick populations (Taylor, 2001). However, these chemical acaracides are environmentally damaging and often toxic to humans and other beneficial organisms. Alternatives to harsh chemical treatment include entomopathogenic fungi and bacteria, and natural predators such as beetles, spiders, and ants but these also have their inherent drawbacks making it difficult to develop an effective non-chemical tick management program (Eisler *et al.*, 2003; George, 2000; Kaaya, 2000b; Kaaya & Hassan, 2000; Pegram *et al.*, 2000; Samish, 2000; Samish *et al.*, 2004).

Beauveria bassiana and other entomopathogenic fungi have been used for the control of insects that harbor disease vectors such as mosquitoes and ticks; against agricultural pests such as whiteflies, caterpillars, grasshoppers, and borers; and against urban pests such as ants and termites (Cruz *et al.*, 2006; Reithinger *et al.*, 1997; Scholte *et al.*, 2004; Scholte *et al.*, 2005). The conidia attach to surfaces by way of cell surface hydrophobicity (Boucias *et al.*, 1988; Drozd & Schwartzbrod, 1996; Holder & Keyhani, 2005; Li *et al.*, 2010). The fungal conidiospores will produce germ tubes which will penetrate into the host insect by physical mechanisms, mycotoxins, secondary metabolites, and proteases, lipases, and chitinases (Alverson, 2003; Clarkson & Charnley, 1996; Kirkland *et al.*, 2005; Stleger *et al.*, 1986). After penetration into the host it will proliferate in the hemolymph as hyphal bodies, colonizing the entire host until

conidiogenesis occurs. Death of the host is due to colonization of the insect haemolymph, tissue damage, and nutrient depletion (Boucias & Pendland, 1991).

Oxalate as an Acaracidal Virulence Factor

Beauveria bassiana is known to secrete an array of extracellular enzymes such as proteases, glycosidases, lipases and toxic metabolites during the infection process (Clarkson & Charnley, 1996; Gupta *et al.*, 1992; Kucera & Samsinak.A, 1968; Stleger *et al.*, 1986). Metabolic acids have been shown to mediate virulence towards some species of grasshopper (Bidochka & Khachatourians, 1991). Oxalic acid $(COOH)_2$ is made by plants, is a major organic acid secreted by several fungi (Gadd, 1999; Kubicek, 1987; Munir *et al.*, 2001), and is a divalent cation chelator secreted during fungal metabolism. This acid has pKa values of 1.3 and 4.3 acting as a source of both protons and electrons which makes it a potent virulence factor for both phytopathogenesis and entomopathogenesis (Alverson, 2003; Guimaraes & Stotz, 2004). It is synthesized via two major pathways, either from glyoxalate or L-ascorbic acid. Both pathways produce oxaloacetate which is hydrolytically cleaved by the enzyme oxaloacetate acetylhydrolase (OAH) to produce oxalate and acetate (Caliskan & Cuming, 1998; Han *et al.*, 2007). High concentrations of oxalic acid in plants are thought to discourage insect foraging and have been shown to be toxic to honey bees and other plant bugs (Alverson, 2003; Franceschi & Horner, 1980; Gregorc & Poklukar, 2003; Horner & Zindlerfrank, 1980; McConn & Nakata, 2002; Nakata, 2002). Oxalic acid also plays a key role in the lignolytic activity and disruption of the plant cell wall of phytopathogenic fungi (Aguilar *et al.*, 1999; Munir *et al.*, 2001).

Several pathways exist in fungi for oxalic acid biosynthesis. In *Aspergillus niger*, oxaloacetate hydrolase can catalyze the conversion of oxaloacetate to oxalate and

acetate (Kubicek, 1987), whereas species of the phytopathogenic fungus *Sclerotium* can oxidize glyoxylate via the activity of a glyoxylate dehydrogenase (Balmforth & Thomson, 1984; Maxwell & Bateman, 1968). These systems link oxalic acid production to the tricarboxylic acid (TCA) and glyoxylate cycles, respectively. However, *A. niger* also possesses both a cytoplasmic pyruvate decarboxylase and oxaloacetate acetylhydrolase that would be capable of forming oxalic acid without the reactions of the TCA cycle (Kubicek *et al.*, 1988). In wood-rotting fungi such as *Fomitopsis palustris* Gilbn. and Ryv., at least two additional oxalic acid-yielding routes, glyoxylate oxidase/oxaloacetase) and a flavohemoprotein glyoxylate dehydrogenase, have been described (Munir *et al.*, 2001). Although the oxalic acid biosynthetic pathway in *B. bassiana* remains to be elucidated, preliminary mapping experiments have indicated the putative presence of at least the cytoplasmic pathway similar to that described above for *A. niger* (Cho and N.O.Keyhani., unpublished data).

Materials and Methods

Ticks

Adult and nymphal ticks *Amblyomma americanum, Amblyomma maculatum, Dermacentor variabilis, Rhipicephalus sanguineus*, and *Ixodes scapularis* were obtained from the Department of Entomology, Oklahoma State University Tick Rearing Facility (Stillwater, OK).

Fungal Cultivation and Maintenance

B. bassiana (ATCC 90517) isolated from *Dysdercus sp*. in Peru (Gupta *et al.*, 1992) and *Metarhizium anisopliae* (ATCC 20500), a soil isolate from Japan, were grown on potato dextrose agar (PDA) or Sabouraud dextrose + 0.5% yeast extract on either agar plates (SDAY) containing 5 µg/ml trimethoprim, or in liquid broth (SDY). Agar

plates were incubated at 26° C for 10-12 days and aerial conidia were harvested by flooding the plate with sterile deionized H_20 containing 0.01% Tween20. Conidial suspensions were filtered through glass wool and final concentration determined by direct count using a haemocytometer. Liquid broth cultures were inoculated with conidia harvested from plates to a final concentration of 0.5-5 x 10^5 conidia/ml. Cultures were grown for 3-4 days at 26° C with aeration. Cultures were filtered through glass wool or Miracloth to remove mycelia, and the concentration of blastospores was determined by direct count. Filtered cell suspensions were harvested by centrifugation (10,000g, 15 min, and 4° C), washed two times with sterile deionized H_2O + 0.02% Tween20, and resuspended to a concentration of 10^8 blastospores/ml. Serial dilutions were made into deionized H_2O containing 0.01% Tween20. The culture supernatant (spent media) was filtered through a 0.22 µm sterilization membrane and added back to harvested cells as indicated.

Bioassays

B. bassiana/ M. anisopliae: Fungal virulence towards ticks was determined using suspensions of varying spore concentrations of either plate harvested or liquid broth grown cells. Ticks were submerged for ~30 sec in spore suspensions (ranging from 10^4-10^8 cells/ml) and the excess fungal suspension removed with either a pipet or a cotton swab. Sterile deionized H_2O containing 0.01% Tween 20 was applied to control ticks. Each trial included 20-50 ticks, and trials at each concentration replicated three times. Ticks were placed in microtiter plates containing numerous needle-puncture holes (to allow for free-flow of air exchange) and stoppered with Styrofoam plugs (Fig 1-2). Specimens were placed in a humidity chamber (>90% RH) with a 12 hour day (27° C)/ night (25° C) cycle, and the ticks were periodically examined microscopically for

fungal growth with mortality recorded every 2-3 days. Tick mortality data were analyzed by PROC MIXED in SAS by using a linear mixed model. The least significant difference (LSD) test was conducted for comparisons between treatments and control inoculations (Kuel, 2000).

Scanning Electron Microscopy (SEM)

Infected adults were examined by scanning electron microscopy (SEM) throughout the time course of the experiment. Ticks were treated with 10^8 conidia/ml *B. bassiana* (replicates=3-5 for each time point) and were examined at 24, 48, and 72 hour, as well as 7 and 14 days post infection on both dorsal and ventral mounts. In instances where some mortality had occurred, both living and dead specimens were processed. Ticks were fixed in 6% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) buffer at 4^{o} C. Ticks were then washed with deionized H_2O and dehydrated in a graded series of ethanol to absolute ethanol before treatment with hexamethyldisilazane for 30 min. Mounted samples were subsequently sputter-coated with 30 nm of gold/palladium and observed using a Hitachi S-570 scanning electron microscopy at 20KV.

Oxalic Acid Virulence Assays

Oxalic acid (>99% purity) and other chemicals and reagents were purchased from Sigma (St. Louis, MO) and fisher (Pittsburgh, PA). Where indicated, culture supernatants (6 day) were treated with proteinase K (MP Biomedicals, Aurora, OH) as follows: samples (2 ml) were incubated with 100 µl of 10 mg/ml proteinase K solution (dissolved in dH_20) for 1 hour at 37^{o} C. Ticks were submerged for ~60 sec in experimental solutions, and excess liquid was removed with either a pipet or a cotton swab. Test solutions included, *B. bassiana* culture supernatants, 50 mM sodium oxalate adjusted to pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 by using either NaOH or

HCl, and 1, 5, 10, 20, and 50 mM solutions of sodium oxalate, sodium formate, sodium phosphate, and sodium citrate adjusted to pH values of 7.0 and pH 4.0 with either NaOH or HCl. Each experiment included 20-50 ticks, and experiments were repeated three times. Ticks were placed in conical tubes or microtiter plates containing numerous needle puncture holes (to allow for free flow of air exchange) and stoppered with Styrofoam plugs. Specimens were placed in a humidity chamber (>90%RH) with a 12 hour day (27^{o} C) /night (25^{o} C) cycle, with mortality recorded every day.

Aliquots of cell-free culture supernatants were analyzed for carbohydrates and organic acids by high performance liquid chromatography (HPLC, HP Series II 1090, Hewlett Packard/Agilen, Wilmington, DE) by using an Aminex HPX-87H column (Bio-Rad) run isocratically in a 4 mM H_2SO_4 and coupled to both UV and refractive index detectors. The oxalate peak was quantified using a standard curve generated using the chemical compound. An aliquot of each supernatant was filtered through a 5,000 MW cutoff membrane (VivaScience, Binbrook Hill, Lincoln, UK), and the filtrate was acidified by addition of dilute sulfuric acid before injection (10-20 µl) onto the column.

Chemical mutants of *B. bassiana* strain 90517 were produced using the alkalyating reagent ethyl methanesulfonate (EMS) essentially as described by St. Leger et.al. (1999). Briefly, a spore suspension (0.1 ml of 1-5 X 10^{7} conidia/ml) was added to 0.9 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 10 µl of EMS. Cells were incubated at 26^{o} C for 5-8 hour with aeration. Samples (0.1-0.5 ml) were diluted 1:10 into buffer containing 10% (wt:vol) sodium thiosulfate and incubated for an additional 0.5-1 hour with aeration. Samples were diluted (typically to 0.5-1 X 10^{3} viable cells/ml) and plated onto selection media (SDY containing 0.01% bromocresol purple;

adjusted to pH 6.8). Plates were incubated at 26^{o} C for 8-10 days. Under these conditions, the wild-type *B. bassiana* strain produced yellow halos within 3-5 days (Fig. 1-12). Colonies that lacked or had reduced zones of yellow (on pH 6.8 plates) were removed, and single spores were isolated and screened on solid pH-indicator media over several generations (three to five times).

Results

Fungal Pathogenicity to *Amblyomma maculatum* and *Amblyomma americanum* Adults and Nymphs

Unfed adult *A. maculatum* were susceptible to the entomopathogenic fungi *B. bassiana* and *M. anisopliae* in a dose-dependent manner (Fig. 1-3). *B. bassiana* conidia harvested from plates appeared to be more infectious against *A. maculatum* than *B. bassiana* blastospores harvested from liquid cultures. Fungal mediated mortality (55%, 28 days post-infection) towards *A. maculatum* was observed at concentrations as low as 10^{6} conidia/ml, with nearly 100% mortality at 10^{8} conidia/ml (Fig. 1-3B). Washed *B. bassiana* blastopores, however, caused little (7%) mortality at 10^{6} cells/ml, but approached that of conidia harvested from agar plates at higher cell concentrations (10^{8} cell/ml, Fig. 1B). *M. anisopliae* conidia harvested from plates resulted in mortality against *A. maculatum* similar to that seen using *B. bassiana* blastospores. These data indicate that a critical concentration threshold of fungal cells (10^{8} cell/ml) appears to be required for high mortality.

B. bassiana conidia had less than 6% mortality against *A. americanum* (28 days post-infection). Low mortality (17%, 28 days post-infection) was also observed using *B. bassiana* blastospores, and widely variable mortality was observed using *M. anisopliae* conidia (17% ± 15% SE).

A time course of the percent mortality using 10^8 fungal cells/ml against *A. americanum* and *A. maculatum* is given in Table 1. The majority of the fungal induced mortality observed towards *A. maculatum* appeared to occur between 14 and 21 days. In all instances adult ticks treated with buffer control resulted in less than 5% mortality over course of the experiment.

The susceptibility of *A. americanum* and *A. maculatum* nymphs was tested using *B. bassiana* and *M. anisopliae* conidia isolated from plates at 10^8 conidia/ml (Table 1-1). *A. americanum* nymphs were more susceptible to both *B. bassiana* and *M. anisopliae* conidia (20–35% mortality) than adults, although mortality remained far lower than that observed against *A. maculatum* (nearly 100% mortality). *A. maculatum* nymphs were far more susceptible to the fungi than *A. americanum* nymphs or adults or *A. maculatum* adults, with 60% mortality observed 7 days post-infection with *B. bassiana* conidia (as compared to 10% mortality in similarly treated adults).

The addition of nutrients (supplement resulting in a final concentration equivalent to 10-fold dilution of Sabouraud media) to fungal cells washed into sterile dH_2O + 0.01% Tween20 did not greatly affect the resultant mortality against either *A. maculatum* or *A. americanum* (Table 1-2). A noticeable reduction in mortality was observed when using *B. bassiana* conidia harvested from plates (95% mortality) as compared to the same cells supplemented at 1:10 Sabouraud media (50% mortality).

Mortality (60–70%) towards *A. americanum* was observed using *B. bassiana* blastospores harvested from broth culture (3–4 days, SDY, Table 1-2). Broth cultures were filtered through glass wool to remove large aggregates and mycelium, resulting in a mixture of newly formed blastospores (70–80%) and residues of conidiospores (20–

30%) used to initially inoculate cultures. Reduced virulence was observed when using washed blastospores, which could be restored by the addition of spent media, indicating that the harvesting and washing procedure did not have a deleterious effect on the blastospores. The observed mortality resulting from use of blastospores directly from the media appeared not to be due to the availability of nutrients, since supplementation of washed fungal blastospores with SDY media did not result in an increase in mortality (Table 1-2). These data indicate that secreted factor(s) found in 3–4 days spent media culture broth appear to enhance *B. bassiana's* virulence towards *A. americanum* ticks.

Conidial Germination on Tick Cuticle

A qualitative comparison of conidial binding and germination of *B. bassiana* on *A. maculatum* and *A. americanum* was performed using scanning electron microscopy of ticks infected throughout the time course of infection (Fig. 1-4). Conidial germination occurred on both tick species, although a greater number of germinating conidia and mycelial formation was visible earlier on *A. maculatum* than on *A. americanum*. Examination of fixed samples indicated that conidial density and germination varied dramatically by body region. Within 72 hours of inoculation, most (germinating) conidia were found in the marginal groove and marginal body fold as well as around the anus and anal groove. In the early stages of infection (*A. maculatum*) comparatively few conidia were observed on the scutum, although patches of fungi could be found within the cervical groove and lateral carina. In several instances both *B. bassiana* and *M. anisopliae* were observed proliferating (in patches) on the cuticle surface of *A. americanum* ticks, although the extent was far lower than that observed for *A. maculatum* and several specimens contained hardly any germinating cells.

Single colony isolates of fungi from *A. maculatum* and *A. americanum* were identified by PCR amplification and cloning of ribosomal RNA gene fragments. Sequencing results of a portion of the 18S rRNA gene and the 5.8S rRNA with its flanking internal transcribed spacer sequences (ITS) were found to be 100% identical between the tick fungal isolate and the stock fungal strain used.

Effect of Cuticular Lipids on Conidial Germination

Pentane extracts of whole cuticular lipids derived from *A. maculatum*, spotted on glass slides produced ~80% spore germination, similar to control assays with no epicuticular extract added (Table 1-3). In contrast, conidial germination was less than 20% in assays performed using *A. americanum* extracts (see Fig. 1-5). Of those conidia that had germinated, the average germ tube length of *A. maculatum* exposed, germinating conidia was more than four times greater than their *A. americanum* counterparts (18 µm versus 4 µm, Table 1-3). Control experiments plating the same batch of *B. bassiana* conidia on nutrient agar (SDAY) resulted in greater than 98% germination within 24 hour, with conidia harvested using sterile dH_2O and spotted onto glass slides, displaying greater than 60% germination after 36 hours even without the addition of any nutrients or media.

Pathogenicity Towards Ixodidae Tick Species

Infection assays using fungal cell suspensions of *M. anisopliae* and *B. bassiana* washed into sterile dH_2O containing 0.01% Tween20 resulted in significant mortality toward *R. sanguineus* (≈70%, 28 days post infection) and *I. scapularis* (65% mortality) in a dose-dependent manner, but only limited virulence against *D. variabilis* (≈10%) (Fig. 1-6). Both blastospores (produced predominantly in Sabouraud dextrose broth, 70–80% blastospores) and conidia (isolated from Sabouraud dextrose agar plates,

>95% conidia) were prepared and used as inocula on the ticks. Only small differences in virulence were observed between the *B. bassiana* blastospores and conidia or between *B. bassiana* and *M. anisopliae.* Experiments to determine the dose dependence of fungal virulence against the tested tick species indicated that a critical threshold of fungal cells (10^8 cells/ml) was required for mortality (>50%) in adult *R. sanguineus* and *I. scapularis* (Fig. 1-6). Mortality within a control group, inoculated with sterile dH_2O containing 0.01% Tween20 was less than 5 ± 2%.

A time course of the mortality measured every 7 days using 10^8 fungal cells /ml as inoculum indicated that for the susceptible species (*R. sanguineus* and *I. scapularis*), significant mortality required at least 14 days of infection, with ≈50% of the overall mortality occurring 14–21 days postinfection (Fig. 1-7). Fungal mycelial outgrowth was visible 21 days post infection on (dead) ticks (Fig. 1-9).

R. sanguineus nymphs were much more susceptible to fungal infection and subsequent mortality than their respective adults (using *B.bassiana* conidia, X=37.03, df=1, P<0.001; using blastospores, X=17.62, df=1, P<0.001). Both *B. bassiana* (10^8 conidia/ml) and *M. anisopliae* (10^8 conidia/ml) resulted in >60% mortality within 14 days, and >90% mortality within 21 days postinfection against *R. sanguineus* nymphs (Fig. 1-8). *D. variabilis* nymphs also seemed to be more susceptible to fungal infection by *B. bassiana* conidia (but not blastospores) than their respective adults (using *B. bassiana* conidia, χ^2 = 9.52, df = 1, *P* = 0.002; using blastospores; χ^2 = 2.53, df = 1, *P* = 0.1114); however, mortality remained low (15–45%, 28 day postinfection). In contrast, *I. scapularis* nymphs did not seem to be any more susceptible to the fungi than conspecific adults (Figs. 1-7 and 1-8).

Mortality (≈65%) was observed in adult *D. variabilis* infection assays only when *B. bassiana* cells were applied to the ticks directly from the broth culture, i.e., with culture supernatant. Virulence decreased by washing the blastospores, but could be restored by suspension in spent media (Table 1-4). The data indicated differences ($P < 0.001$) for comparisons between the treatment (unwashed blastospores or washed blastospores supplemented with spent broth) and control inoculations with deionized H_2O or Sabouraud broth, inoculations using 10^7 conidia/ml or 10^8 conidia/ml (washed into dH_2O), and washed blastospores. The reduced mortality of blastospores towards *D. variabilis* was not due to availability of nutrients because fungal cells supplemented with Sabouraud media (1:10) did not result in any significant increase in mortality (Table 1-4). These data indicate that secreted factors found in spent culture supernatant caused virulence toward *D. variabilis.*

Ticks were examined throughout the 28 day time course of the infections by scanning electron microscopy (Fig. 1-4 & 1-9). Comparable concentrations of conidia were visible 1–12 hour postinfection on all three ticks species tested, although the distribution of conidia was not uniform across the body of the ticks. Conidial germination and proliferation were much more evident on both *I. scapularis* and *R. sanguineus* ticks during the first week postinoculation, than on *D. variabilis* ticks, although a wide variation was observed. Germination of most of the bound conidia was visible within 24–48 hour postinfection. Hyphal growth was evident 2–14 day postinfection, although clear instances of appressoria or penetration events were difficult to distinguish on the surface of the tick. Extensive fungal growth was visible on several distinct regions of the tick anatomy including the anal and genital grooves and apertures, and on the

alloscutum. Patches of fungal growth also could be seen on the capitulum, especially around the mouthparts, and scattered around the idiosoma, particularly within the various lateral and marginal grooves along the surface contours of the organism. In several instances, bacteria or other fungi, clearly distinguishable from the inoculated organism (*B. bassiana*) could be seen on the ticks. *B. bassiana* cells were observed on living *D. variabilis* ticks throughout the time course of the experiments performed, including at the 28 day time point. Conidial binding, germination, and even mycelial growth was apparent on the surface of *D. variabilis* ticks, indicating that the observed low mortality rate may be due to inhibition of critical events required for penetration of the cuticle.

Oxalate as an Acaracidal Virulence Factor

Our previous results had indicated that addition of spent culture supernatant could increase fungal mediated mortality toward certain tick species (Kirkland *et al.*, 2004a; Kirkland *et al.*, 2004b). Adult *A. americanum* were susceptible to cell-free culture supernatants derived from growth of the entomopathogenic fungi *B. bassiana* in Sab or SDY (Table 1-5). Mortality was observed within 14 day by using fungal spent culture supernatant isolated from 6 day cultures grown in Sab (20%) or SDY (50%) media, with lower mortality seen using spent PD media (12%), and little to no mortality observed using culture supernatants from CzD media (<6%). A second treatment with Sab or SDY spent media applied 14 days after the initial treatment resulted in up to 65–85% (total) mortality within 28 days of the original treatment, whereas second applications of spent PD or CzD media did not result in any increased mortality. Similar experiments treating adult *A. maculatum* or *I. scapularis* ticks with SDY supernatants resulted in 50 ± 10 and 32 ± 15% (14 days post treatment) mortality, respectively. Control treatments

with sterile media or dH_2O resulted in less than 5% mortality throughout the time course of the experiments. To test the heat lability of the observed acaricide activity, aliquots of 6 day Sab or SDY cell free culture supernatants were boiled for 10 min, allowed to cool to room temperature for 10 min, briefly spun to remove any precipitation, and then used to treat adult *A. americanum* ticks. Boiled supernatants resulted in 18 ± 5 (Sab) and 40 ± 10% (SDY) mortality after 14 days. To test whether the acaricidal activity was primarily proteinaceous in nature, aliquots of Sab and SDY culture supernatants treated with proteinase K (1 mg/ml) for 1 hour at 37° C resulted in 24 ± 6 and 50 ± 10% mortality (14 day post treatment). By contrast, dialysis using a 10,000-molecular weight cut-off membrane of active culture supernatants against 50 mM Tris buffer, pH 7.0 (10 ml aliquot versus 2 by 2 liters, overnight), resulted in a reduction in the acaricide potency of the culture supernatants, with total mortality percentages dropping to 5 ± 3% for SD and 9 ± 3% for SDY media, 14 days post treatment of adult *A. americanum* ticks. These findings suggest that *B. bassiana* secretes a small heat-stable acaricide compound into Sab and SDY broth during growth. Acaricide activity was stable to repeated freeze thawing cycles (at least three) and at 4°C for a least 1 month.

Analysis of cell free culture supernatants by HPLC revealed oxalic to be the major organic acid present with minor amounts of formate, citrate and acetate also detected. The oxalate concentrations as well as the pH of the culture supernatants used to treat the ticks were determined (Table 1-5). The concentration of oxalic acid and the resultant decrease in pH correlated with the acaricide activity of the culture supernatants. Oxalic acid at concentrations of 20–35 mM were produced when the fungal cells were grown in Sab or SDY media, whereas lower amounts of oxalic acid

(≈10 mM) were secreted when the cells were grown in PD, and almost no oxalate was produced when the cells were grown in CzD (<0.5 mM) under the conditions tested.

The acaracidal toxicity of oxalic acid was tested by using the chemical compound to treat adult *A. americanum* ticks (Fig. 1-10). Greater than 60% (14 days post treatment) and ≈20% mortality was observed using 50 and 20 mM oxalic acid, pH 4.0, respectively. No significant mortality was observed using a single treatment with lower concentrations of oxalate (1–10 mM), pH 4.0, or with solutions of oxalate at pH 7.0 (1–50 mM). Furthermore, no acaracidal activity was observed using single treatments of solutions of either of citrate, formate, or phosphate at pH values of either 4.0 or 7.0 and using concentrations up to 50 mM (Fig. 1-10). A second treatment 21 days after the initial treatment resulted in 75–85% total mortality by using either 20 or 50 mM oxalate, pH 4.0, and ≈40% mortality by using 50 mM oxalate, pH 7.0, within 14 days (35 days total).

The pH dependence of the acaracidal activity of oxalate was investigated using oxalic acid solutions ranging from pH 4.0 to 7.0 (Fig. 1-11). Acaracidal activity was highest at pH <4.0 (>80% mortality, 14 days post treatment) with tick mortality rapidly decreasing as the pH of the oxalate solution was raised. Mortality at pH 4.5 was four-fold lower than that observed for solutions of oxalate at pH 4.0. Second treatments, 14 days after the first, had a minor effect, resulting in up to 40% total mortality.

To assess whether oxalic acid production by *B. bassiana* was indeed a contributing factor in the acaracidal activity of culture supernatants, mutant screens were established to isolate oxalic acid nonproducers. Surviving colonies of EMS-treated conidia were plated onto SDY media supplemented with the pH indicator dye

bromocresol purple ($pH_{initial}$ 6.8). Approximately 5,000 mutant clones of *B. bassiana* strain 90175 were screened on the indicator plates, and six mutants were identified that lacked or had reduced zones of surrounding yellow (acidification) (Fig. 1-12). Of these, three were shown to be false positives due to production of wild-type like yellow zones when single spores were isolated and rescreened. The remaining three mutants, designated as clones A1 + 15, A1 + 16, and A1 + 17, seemed to retain their phenotype (no yellow zone of clearing, purple colonies) after at least three generations of rescreening on the indicator plates. Mutants A + 15 and A1 + 16 displayed altered conidiation effects, forming smaller colonies that grew slower but sporulated more rapidly than wild type on PDA, Sab, and SDY agar plates. Mutant A1 + 17 also displayed altered colony morphology but sporulated poorly when grown on PDA, Sab, or SDY agar plates. The concentration of oxalic acid secreted by the mutants was quantified over a 15 day time course of growth in SDY broth (Fig. 1-13). These data indicated that two of the isolates, A1 + 15 and A1 + 16 produced no detectable oxalic acid under the conditions tested. Interestingly, the third mutant (A1 + 17) was able to produce oxalic acid (~12 mM) at approximately one-half the levels as that of the wild-type strain. Culture supernatants (day 6) from the three mutants were used to treat adult *A. americanum* ticks in mortality experiments as described previously. Less than 10% mortality toward *A. americanum* ticks was observed using culture supernatants derived from any of the mutants grown in SDY including, A1 + 15, A1 + 16, and A1 + 17, even though the oxalic acid concentration in the latter supernatant approached 12 mM.

Discussion

Pathogenicity

B. bassiana and *M. anisopliae* strains have been used for control of insect pests in agricultural, ecological, and domestic settings in a number of countries (Ferron, 1981; Leathers *et al.*, 1993; McCoy, 1990; Roberts & Humber, 1981). The use of these organisms in the biocontrol of arthropod pests, such as ticks, is gaining impetus. The pathogenicity of entomopathogenic fungi to different developmental stages of *R. sanguineus* has been investigated (Samish *et al.*, 2001). In these studies, *M. anisopliae* was the most virulent isolate, with *B. bassiana*, *M. flavoviride*, and *Paecilomyces fumosoroseus* strains resulting in significantly lower mortality under the conditions tested. The virulence of *M. anisopliae* also has been tested against unfed and engorged adult *R. appendiculatus.* Fungal concentrations as low as 10^6 spores/ml caused 35% (unfed) and 80% (engorged) mortality in these ticks, respectively (Kaaya & Hassan, 2000). Similarly, almost 100% mortality has been shown using *M. anisopliae* at high conidia concentrations (4×10^9 spores/ml) against unfed adult *I. scapularis*, whereas only 10^7 spores/ml induced comparable mortality in engorged adult females (Benjamin *et al.*, 2002; Zhioua *et al.*, 1997). It is likely that increased exposure, and hence susceptibility, occurs during engorgement due to the extension of the cuticle. These data indicate that lower infectious fungal concentrations may be effective in controlling infestations on feeding animals. Some caution, however, should be taken in extrapolating from laboratory results to effective on-host biocontrol. Although, *M. anisopliae* and *B. bassiana* induced high mortality in ticks confined in bags on Zebu cattle ears (Kaaya & Hassan, 2000), a preliminary report using *M. anisopliae* versus *Boophilus microplus* (Canestrini) ticks (unconfined) on stabled bulls, did not result in a

reduction in the total number of ticks that continued to parasitize the animals (Correia *et al.*, 1998).

The effects of *M. anisopliae* also have been determined on non-feeding *I. scapularis* adults (Benjamin *et al.*, 2002). In the field, a mortality of slightly >50% was noted among ticks collected from vegetation plots sprayed with an aqueous formulation of *M. anisopliae.* Here, too, the authors note that their results may contain an upward bias in the mortality rate due to two factors. First, collected ticks were placed in vials under optimum conditions for fungal growth and some may not have died under field conditions; and second, ticks were collectively housed in a single vial/plot, possibly resulting in horizontal transfer of infection from a subpopulation of infected ticks to uninfected ticks.

Factors other than spore concentration that may influence the practical application of pathogenic fungi in tick biocontrol include formulations (aqueous versus oil), fungal growth conditions, and number of applications. For *M. anisopliae,* aqueous formulations resulted in almost 65% mortality in potted grass tetrapacks, whereas oil formulations under identical conditions resulted in >80% mortality (Kaaya, 2000a). Furthermore, the results of this study should be interpreted with the understanding that other *B. bassiana* and *M. anisopliae* isolates may be more or less pathogenic toward ticks and that passage through tick species may select for more virulent or species-specific isolates.

Our results demonstrated dose-dependent mortality toward unfed adult and nymphal *R. sanguineus* and *I. scapularis*, with limited mortality versus *D. variabilis* ticks by using the entomopathogenic fungi *B. bassiana* and *M. anisopliae*, harvested from either agar plates or liquid media and washed into sterile dH_2O containing 0.01%

Tween20. The detergent was used to (i) increase the recovery of fungal cells from agar plates, (ii) decrease aggregation of the isolated conidia, and (iii) ensure a more even distribution of the cells during application. Surface growth of the fungi was observed on treated ticks and both *B. bassiana* and *M. anisopliae* could be recovered from infected specimens, although other fungal species were observed during fungal isolation from tick cadavers. A spore concentration of 10^8 spores/ml was required for effective mortality, presumably to overcome tick defenses during penetration of the tick's cuticle and proliferation inside the host. Significant mortality against *D. variabilis* was observed only when *B. bassiana* blastospores with growth media carryover (i.e., supplemented with spent media) was used as the inoculum. Importantly, addition of fresh media to harvested cells did not induce any increase in mortality under the conditions tested. The most likely explanation for our observations is that *B. bassiana* secretes important virulence factors that can enhance or enable pathogenesis. Growth in various media, including Sabouraud/yeast extract, is known to result in secretion of hydrolytic and proteolytic enzymes, as well as various mycotoxins such as beauvericin and oosporein, and to result in the acidification of the media (Gupta *et al.*, 1992; Kucera & Samsinak.A, 1968; Mazet *et al.*, 1994; St Leger *et al.*, 1997; St Leger *et al.*, 1998). Further study of inoculum conditions to identify important virulence enhancing components may, therefore, lead to improvements in application formulation of these agents.

Differential Susceptibility

Both *B. bassiana* and *M. anisopliae* conidia or blastospores washed into sterile dH_2O were found to be virulent towards *A. maculatum* ticks, but displayed limited mortality towards *A. americanum* ticks. Surface growth of the fungi was observed on treated ticks and both *B. bassiana* and *M. anisopliae* could be recovered from infected

specimens, although other fungal (and bacterial) species were occasionally observed during fungal isolation from tick cadavers. A critical spore concentration was required for effective mortality, presumably to overcome tick defenses during proliferation of the tick cuticle. Moderate mortality towards *A. americanum* was observed only when fungal cells directly from the growth media, i.e., with growth media carry-over were used. Importantly, addition of fresh media to washed fungal cells did not increase mortality towards *A. americanum* (and in some instances appeared to decrease mortality against *A. maculatum*). These data imply that fungal secretion products can help to mediate successful virulence against recalcitrant targets. Both *B. bassiana* and *M. anisopliae* are known to express and secrete a wide variety of compounds including proteases, glycosidases, lipases, peptide mycotoxins, and even organic molecules such as oxalate, all of which have been implicated as pathogenicity factors (Bidochka & Khachatourians, 1991; Gupta *et al.*, 1991; Kucera & Samsinak.A, 1968; Roberts, 1981; St Leger *et al.*, 1997; St Leger *et al.*, 1999). It is possible that secreted factors present when using unwashed fungal cells enables the fungus to overcome defenses found on *A. americanum* but not on *A. maculatum*.

An intriguing possibility is that these secreted factors may assist in overcoming the toxic effects of compounds present in *A. americanum* cuticles. Pentane derived cuticular hydrocarbon extracts of *A. americanum* but not of *A. maculatum* were shown to inhibit germination of *B. bassiana* conidia. Furthermore, of those conidia that had germinated, germ tube formation and hyphal growth was shorter in the presence of *A. americanum* lipid extracts as compared to *A. maculatum* and control samples. An analysis of the cuticular hydrocarbon content of several *Amblyomma* tick species reported the lipid

composition to consist mostly of branched paraffins and olefins, with the number and carbon-chain length of the hydrocarbons distinct between *A. maculatum* and *A. americanum* (Hunt, 1986), although whether any of these differences can account for the observed differences in susceptibility to the entomopathogenic fungi tested is not known.

Several studies have highlighted the complex interaction between cuticular lipids and conidial germination. Germination and hyphal growth of a *B. bassiana* strain virulent towards *Ostrinia nubilalis* but non-pathogenic towards *Melolontha melolontha* occurred in the presence of pentane cuticular extracts of the host insect (*O. nubilalis*) but was inhibited by lipid extracts derived from the non-host insect (*M. melolontha*) (Lecuona *et al.*, 1997). The active compound responsible for hyphal growth inhibition was found mostly in the unsaturated hydrocarbon fraction. Cuticular lipids of the silverleaf whitefly (*Bemisia argentifolii*) were found to inhibit germination of *B. bassiana* conidia on nutrient agar but had no effect on germination rates in the absence of nutrients (James *et al.*, 2003). Fungal germination rates also varied greatly between insect developmental stages, as well as between fungal species. Furthermore, fungal species displayed differential susceptibilities to the effects of lipids due to hydrophobicity. Synthetic long-chain wax esters inhibited conidial germination of *Paecilomyces fumosoroseus* but not of *B. bassiana* (James *et al.*, 2003). Studies on the stinkbug, *N. viridula*, revealed that lipid fractions extracted from the exuviae of the insects inhibited germination of *M. anisopliae* conidia (SosaGomez *et al.*, 1997). In these studies, the aldehyde (*E*)-2-decenal, a primary component of the stick bug scent gland, was detected in the cuticle extracts and found to be fungistatic towards *M. anisopliae*. Further study on tick

cuticular lipid composition and the existence of possible tick defense compounds, as well as on inoculum conditions may identify important virulence modulating components and can lead to rational design strategies for the improvement for the use of fungi as acaricidal biocontrol agents.

Oxalic Acid Acaracidal Activity

Although oxalic acid has been demonstrated to be an important virulence factor for the successful pathogenesis of phytopathogenic fungi during plant host infection, many plant species themselves produce oxalic acid, presumably to discourage insect foraging. Studies using oxalic acid have demonstrated oxalic acid to be toxic toward several insect species, including the tarnished plant bug (Alverson, 2003) and the migratory grasshopper (Bidochka & Khachatourians, 1991). In the latter report, metabolic acids produced by *B. bassiana* (including oxalate and citrate) acted synergistically with fungal conidia to promote successful pathogenesis. With respect to the Acari, oxalic acid has been used to control varroosis, and practical applications have demonstrated reduced infestations of *V. destructor* in bee colonies under conditions that present low toxicity toward the bees themselves (Gregorc & Poklukar, 2003). Interestingly, entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* have demonstrated virulence toward *Varroa* species, and has also been tested for use in control of these mites in bee colonies (Kanga *et al.*, 2002; Kanga *et al.*, 2003).

A. americanum ticks were predominately used in our studies due to the observation that this species can resist fungal (*B. bassiana* and *M. anisopliae*) infection (Kirkland *et al.*, 2004b). Our results indicate that this resistance can be overcome and that (cell-free) culture supernatants derived from the entomopathogenic fungus *B. bassiana* can be toxic toward these ticks (and others), depending upon the fungal

growth and media composition. HPLC analysis of culture supernatants coupled to tick mortality experiments confirmed that one of the major acaricidal active ingredients in these supernatants was oxalic acid, although during fungal infection, secretion of factors such as hydrolytic enzymes, including proteases, glycosidases, and lipases, as well as other biologically active small molecules and toxins undoubtedly contributed to the establishment and progression of disease.

Oxalic acid has a relatively simple chemical formula $(COOH)_2$, that displays at least three important chemical properties: it can act as a proton donor, an electron donor, and as a strong chelator of divalent cations. Our results indicated that oxalate toxicity was pH dependent, with mortality rates dramatically decreasing at pH >4.5 (single application). These data suggest that (as a diprotic compound with pK_a values of 4.28 and 1.29) the reducing potential of oxalate may be an important factor in its tick toxicity. The relatively high concentration of oxalic acid, 50 mM, required for inducing mortality (in single treatments) may suggest that for the fungal organism, oxalic acid acts synergistically with other factors in promoting pathogenesis. Oxalic acid concentrations in culture supernatants did approach 30–35 mM, and it is possible that local oxalic acid concentrations during the infection process could be appreciably higher. Furthermore, hosts are likely to be continuously exposed to the secreted metabolites (including oxalic acid) that are likely to increase their toxicity. Indeed, oxalic acid is able to solubilize several components of insect cuticles, including elastin and collagen, and has been demonstrated to disrupt the integrity of *M. sanguinipes* cuticle directly (Bidochka & Khachatourians, 1991).

Using SDY pH indicator plates, three *B. bassiana* EMS-derived mutants were isolated displaying lowered levels of secreted oxalic acid (two of which produced <1% of the wild-type levels of oxalic acid under the conditions tested). Culture supernatants derived from all three mutants were nontoxic toward *A. americanum* ticks. Although these observations support the hypothesis that oxalic acid is an important fungal virulence factor during pathogenesis toward ticks, some caution should be taken in interpretation. Primarily, oxalic acid may act as a marker for other fungal factors required for pathogenesis, and disruption of pH pathways may have pleiotropic effects. In *M. anisopliae*, oxalic acid production and the resultant reduction in extracellular pH are linked to protease production and activity (St Leger *et al.*, 1999); mutants unable to acidify the media also were deficient in protease activity. This is similar to observations concerning phytopathogenic fungi where the secretion of oxalic acid leads to an acidic environment required for the expression and activities of many hydrolytic enzymes (Bateman & Beer, 1965; Rollins & Dickman, 2001). The virulence of the *B. bassiana* mutants was not assessed directly (i.e., by application of fungal cells to ticks) because these clones probably contain multiple mutations that would have obscured interpretation of any results, particularly because the mutants displayed altered developmental and conidiation phenotypes. Future research using targeted gene knockouts of enzymes in the oxalic acid biosynthetic pathway(s) (see below) of *B. bassiana* will probably help in understanding the physiological role of oxalate during pathogenesis.

Finally, our results indicate that examining inoculum conditions that would favor oxalic acid production could increase the efficacy of field applications of *B. bassiana* in

biocontrol efforts. This increase may be achieved by the selection of oxalic acid-producing constitutive strains, optimization of the conditions for oxalic acid production in already used strains, or even manipulation of dispersion formulas that maximize rapid oxalic acid production.

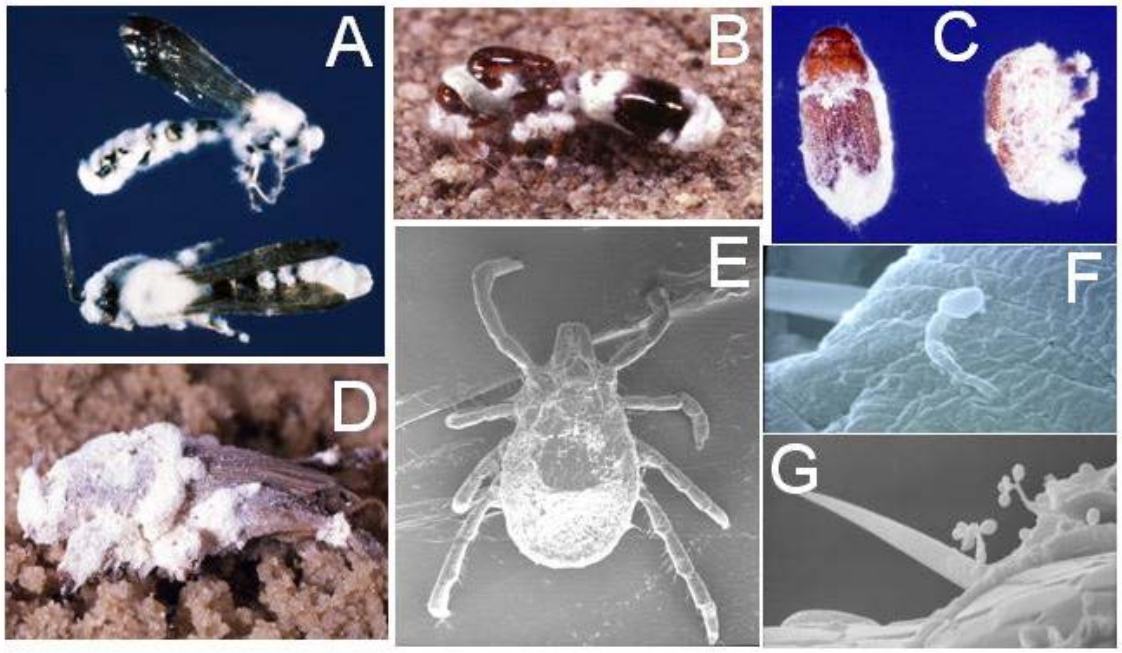


Figure 1-1. B. bassiana has an exceptionally broad host range that spans across Arthropoda classes from insects including; wasps (A), fire ants (B), bark beetles (C), and mole crickets (D) to arachnids such as mites and ticks (E). Cuticle penetration (F), and conidiogenesis (formation of new spores) from host cadaver (G) are also illustrated. (Images A,B,C,D courtesy of D. Boucias).

Figure 1-2. Six well culture plates with stryofoam plugs used for tick bioassays.

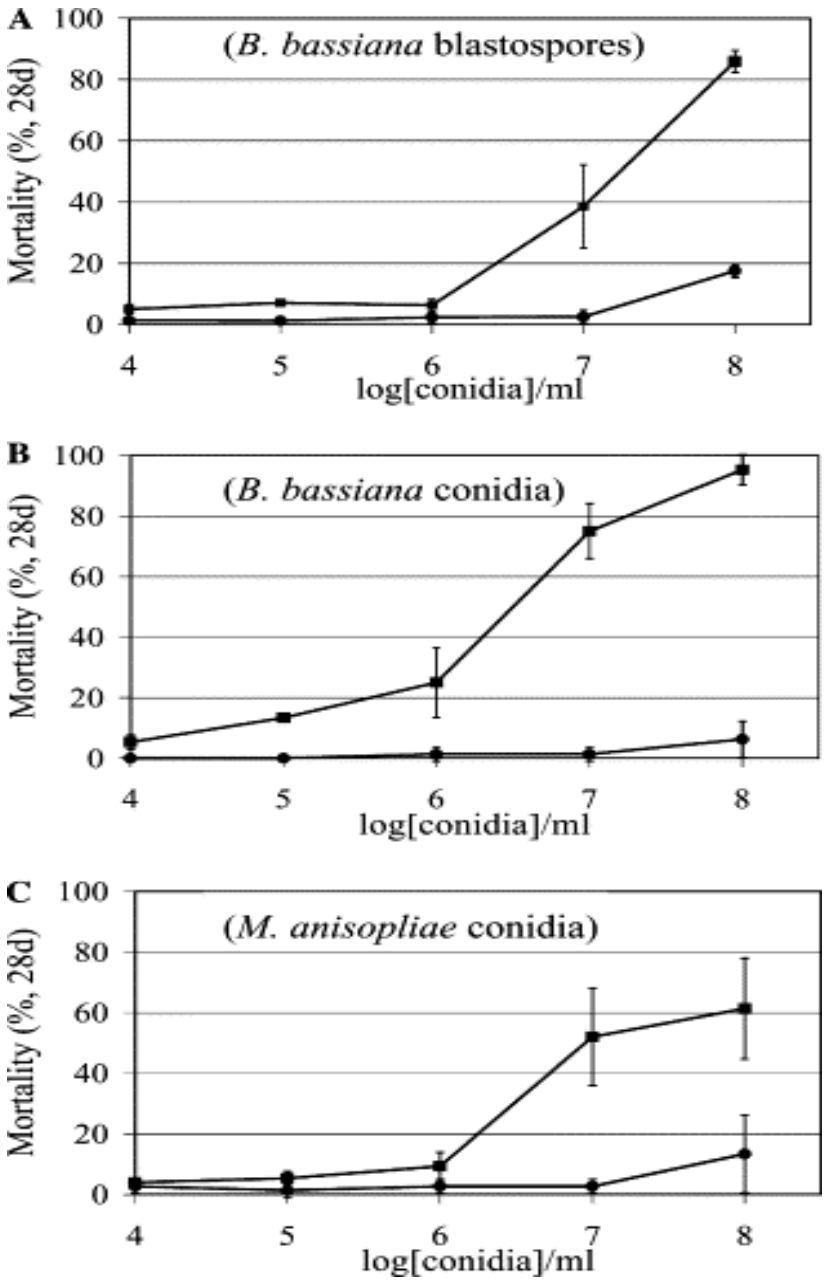


Figure 1-3. Percent mortality 28 days post-infection of unfed adult *A. maculatum* (■) and *A. americanum* (•) treated with *B. bassiana* blastospores (A), *B. bassiana* conidia (B), and *M. anisopliae* conidia (C) as a function of spore concentration. Values given are means of three experiments ± SE.

Table 1-1. Weekly mortality rates for *A. maculatum* and *A. americanum* adults and nymphs treated with fungal suspensions

Treatment	Mortality (%)			
	A. maculatum (adults)[a]	*A. maculatum* (nymphs)[b]	*A. americanum* (adults)[a]	*A. americanum* (nymphs)[b]
B. bassiana (10^8 blastospores/ml)				
7 days post-infection	8 ± 2	65 ± 10	5 ± 3	7 ± 3
14 days	13 ± 3	80 ± 10	10 ± 5	18 ± 5
21 days	64 ± 10	95 ± 5	13 ± 6	21 ± 6
28 days	86 ± 5	98 ± 4	18 ± 2	35 ± 12
B. bassiana (10^8 conidia/ml)				
7 days post-infection	10 ± 3	60 ± 8	0	4 ± 2
14 days	41 ± 5	77 ± 13	1 ± 1	8 ± 3
21 days	85 ± 10	93 ± 7	4 ± 2	16 ± 11
28 days	95 ± 5	95 ± 5	6 ± 5	26 ± 15
M. anisopliae (10^8 conidia/ml)				
7 days	3 ± 1	30 ± 5	1 ± 1	3 ± 2
14 days	19 ± 6	62 ± 12	5 ± 1	8 ± 5
21 days	37 ± 10	88 ± 10	8 ± 1	16 ± 10
28 days	61 ± 17	99 ± 1	13 ± 2	21 ± 2

[a] Mortality of (adult) ticks treated with sterile dH_2O less than 5% throughout the time course of the experiments. [b] Mortality of *A. maculatum* and *A. americanum* nymphs treated with sterile dH_2O reached up to 15 and 6%, respectively, within the time course of the experiments.

Table 1-2. Effect of inoculum composition on *B. bassiana* mediated mortality towards adult *A. maculatum* and *A. americanum.*

Treatment	Mortality (%)	
	A. maculatum	*A. americanum*
10^7 conidia/ml in Sab broth [a]		
7 days post-infection	8 ± 4	3 ± 2
14 days	15 ± 5	4 ± 2
21 days	43 ± 12	5 ± 2
28 days	61 ± 15	9 ± 4
10^8 conidia/ml in Sab broth[a]		
7 days post-infection	7 ± 2	2 ± 1
14 days	13 ± 3	4 ± 2
21 days	32 ± 10	5 ± 2
28 days	69 ± 12	9 ± 3
Washed 10^7 blastospores/ml		
7 days	5 ± 2	0
14 days	12 ± 6	2 ± 1
21 days	19 ± 8	4 ± 2
28 days	32 ± 15	8 ± 2
Unwashed 10^7 blastospores/ml[b]		
7 days post-infection	15 ± 3	11 ± 4
14 days	72 ± 8	45 ± 10
21 days	95 ± 10	55 ± 10
28 days	98 ± 15	70 ± 12

[a] Ticks were inoculated with *B. bassiana* suspensions in 1:10 dilution of Sabouraud broth. Mortality of ticks treated with sterile dH_2O and sterile Sabouraud broth was less than 5 and 10%, respectively, throughout the time course of the experiments. [b] The cell culture was filtered through glass wool to remove mycelial clumps and used directly as inoculum.

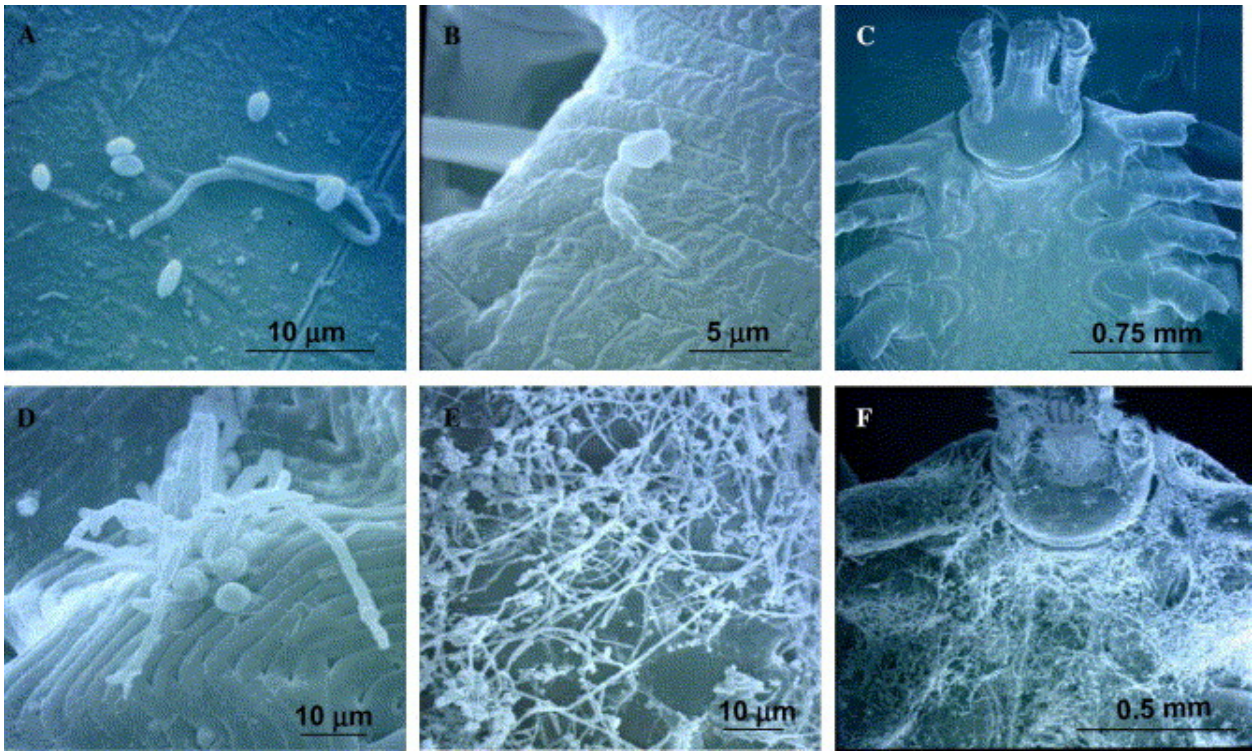


Figure 1-4. Representative electron micrographs of the *B. bassiana* conidia mediated infection process. Conidia bound to *A. americanum* cuticle, 24 hour post-infection (A), 7 days post-infection (B), and 14–21 days post-infection (C). Conidia bound to *A. maculatum* cuticle 24 hour post-infection (D), 7 days post-infection (E), and 14–21 days post-infection (F).

Table 1-3. Effect of cuticular lipid extracts derived from adult *A. maculatum* and *A. americanum* on *B. bassiana* spore germination and germ tube length

Solvent/extract	% spore germination[a]	Mean germ tube length (µm)[b]
Pentane	75 ± 8	14 ± 4
A. maculatum	80 ± 9	18 ± 7
A. americanum	18 ± 10	4 ± 2

[a] Values are expressed as means ± SD using three separate extracts and three to four replicates (each replicate consisting of at least two–three fields of view) for each extract.
[b] The germ tube length of a minimum of 100 germinating conidia for each extract was determined.

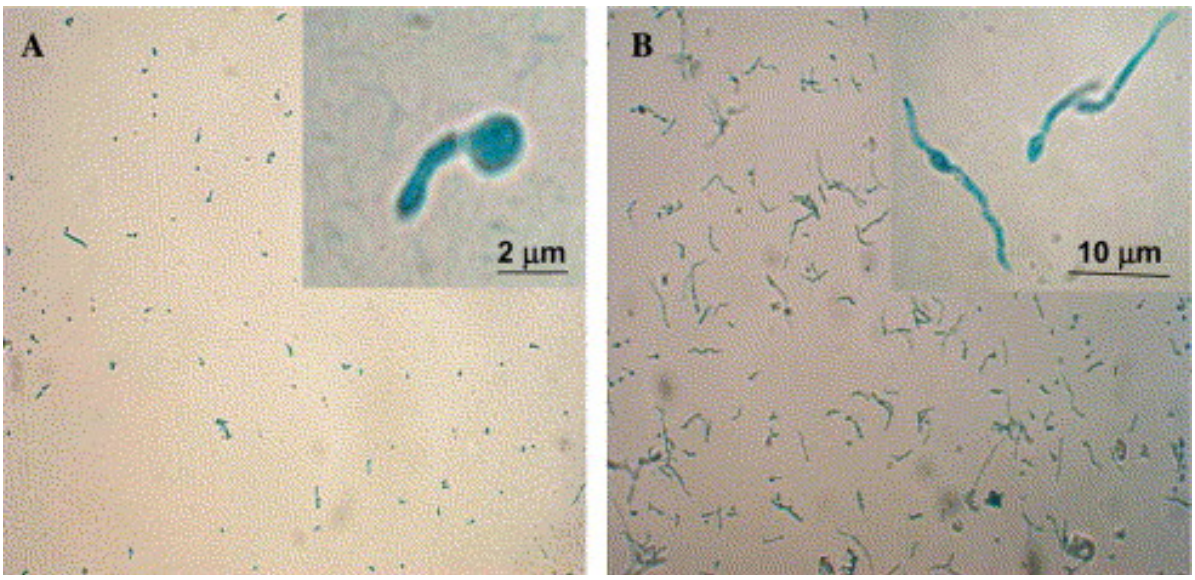


Figure 1-5. *Beauveria bassiana* spore germination on tick cuticular extracts. Conidia were overlaid onto glass slides pre-spotted with pentane extracts of *A. americanum* (A) and *A. maculatum* (B) prepared as described in Section 2. Samples were incubated at 25 °C at 95% humidity for 36 hour before addition of lactophenol blue for fungal cell wall visualization. Insets are higher magnifications of samples illustrating germ tube formation on individual conidia.

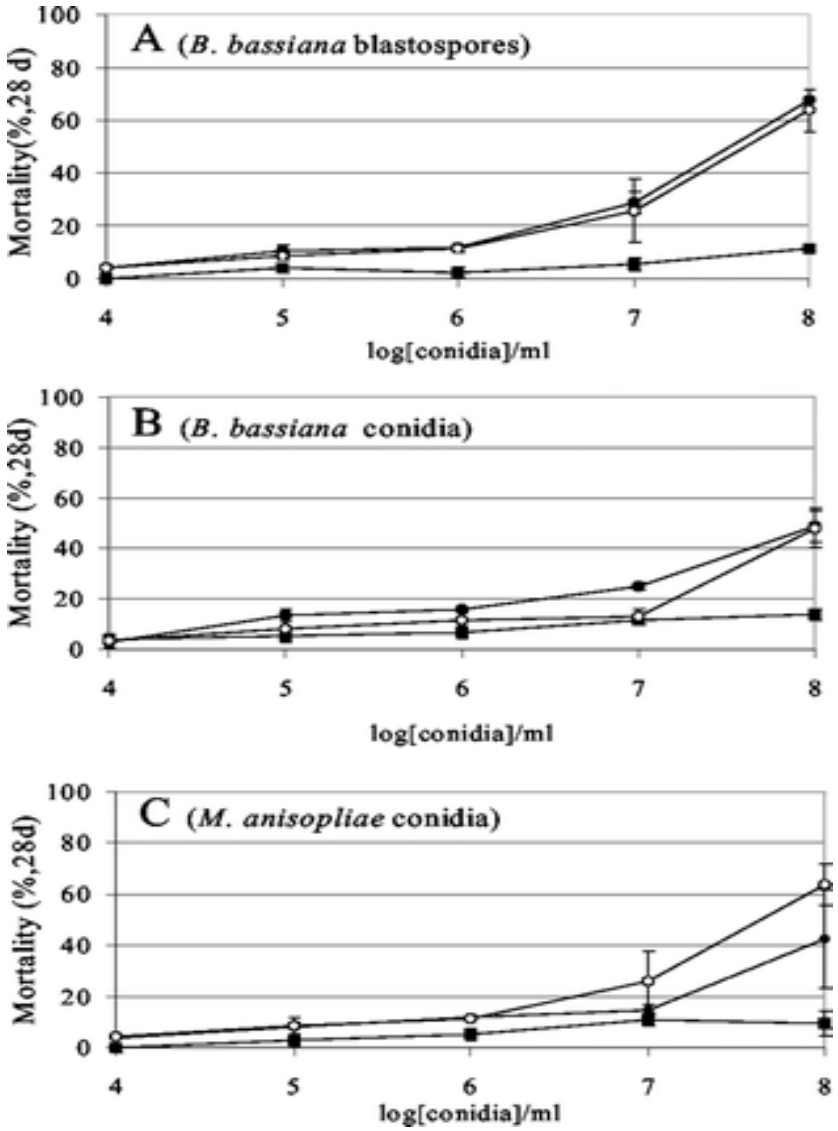


Figure 1-6. Percentage of mortality 28 d postinfection of adult *R. sanguineus* (•), *I scapularis* (○), and *D. variabilis* (▪) inoculated with *B. bassiana* blastospores (A), *B. bassiana* conidia (B), and *M. anisopliae* conidia (C) as a function of spore concentration. Values given are means of three experiments ± SE

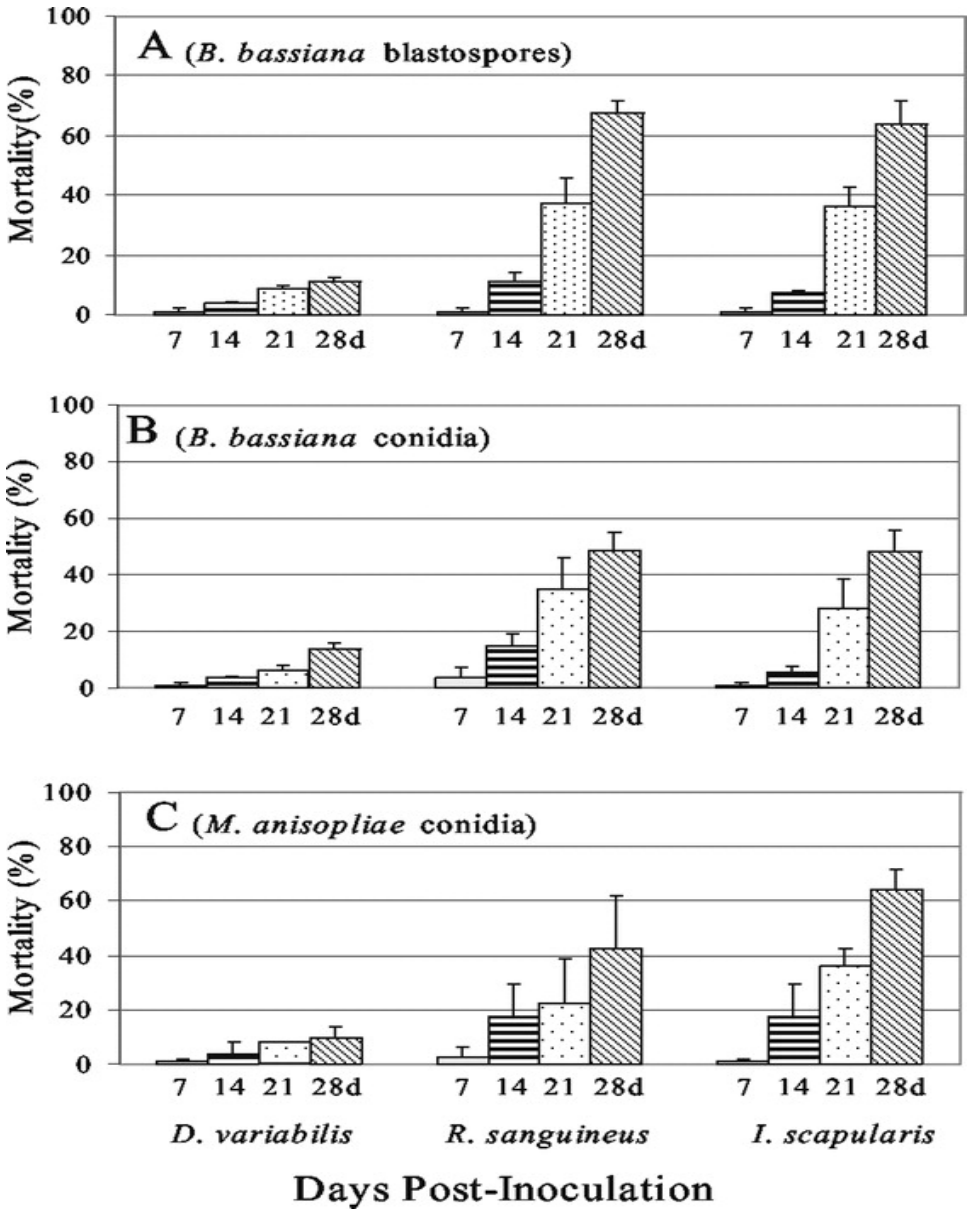


Figure 1-7. Weekly mortality rates for adult *R. sanguineus*, *I. scapularis*, and *D. variabilis*, inoculated with *B. bassiana* blastospores (A), *B. bassiana* conidia (B), and *M. anisopliae* conidia (C) by using 10^8 fungal cells/ml. Values given are means of three experiments ± SE.

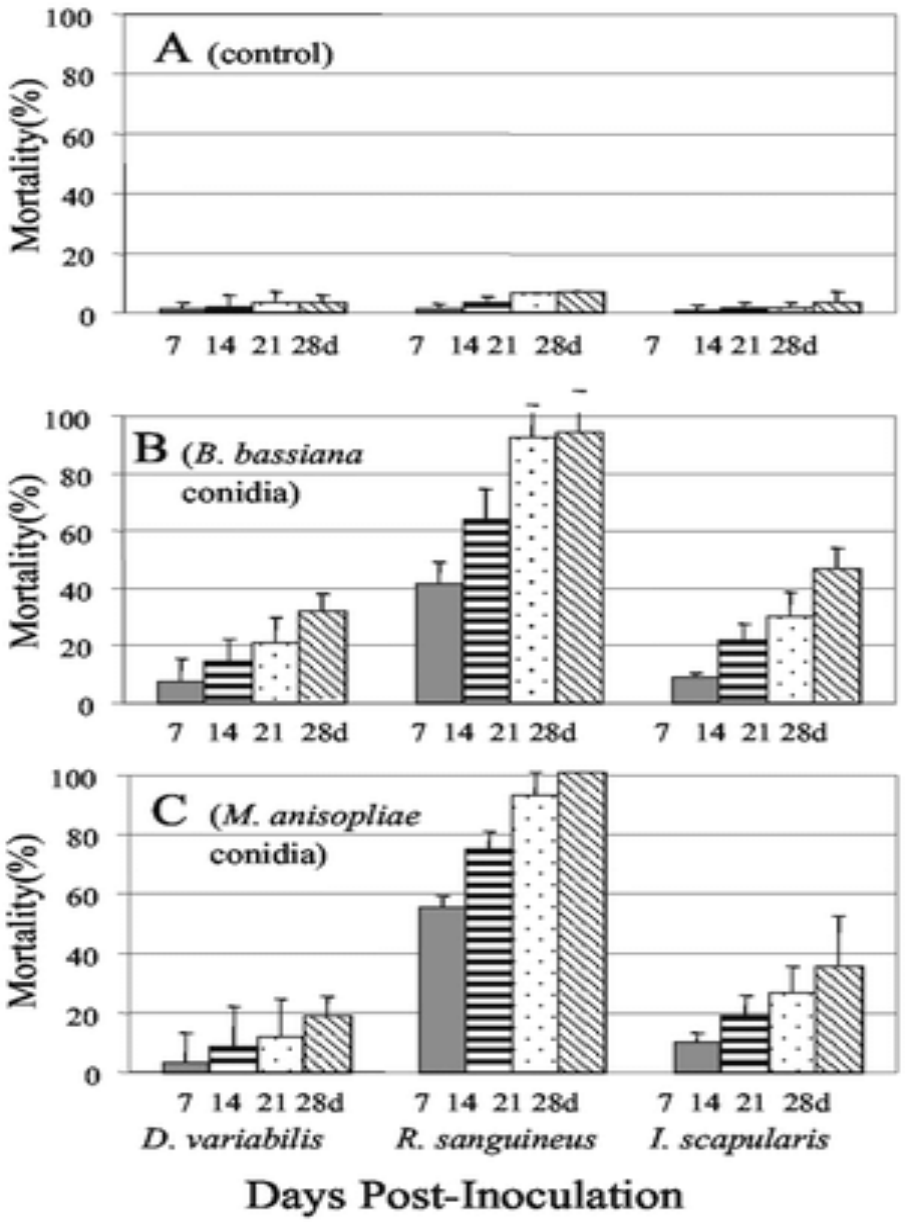


Figure 1-8. Weekly mortality rates for *R. sanguineus*, *I. scapularis*, and *D. variabilis* nymphs inoculated with buffer controls (A), *B. bassiana* conidia (B), and *M. anisopliae* conidia (C) by using 10^8 fungal cells/ml. Values given are means of three experiments ± SE.

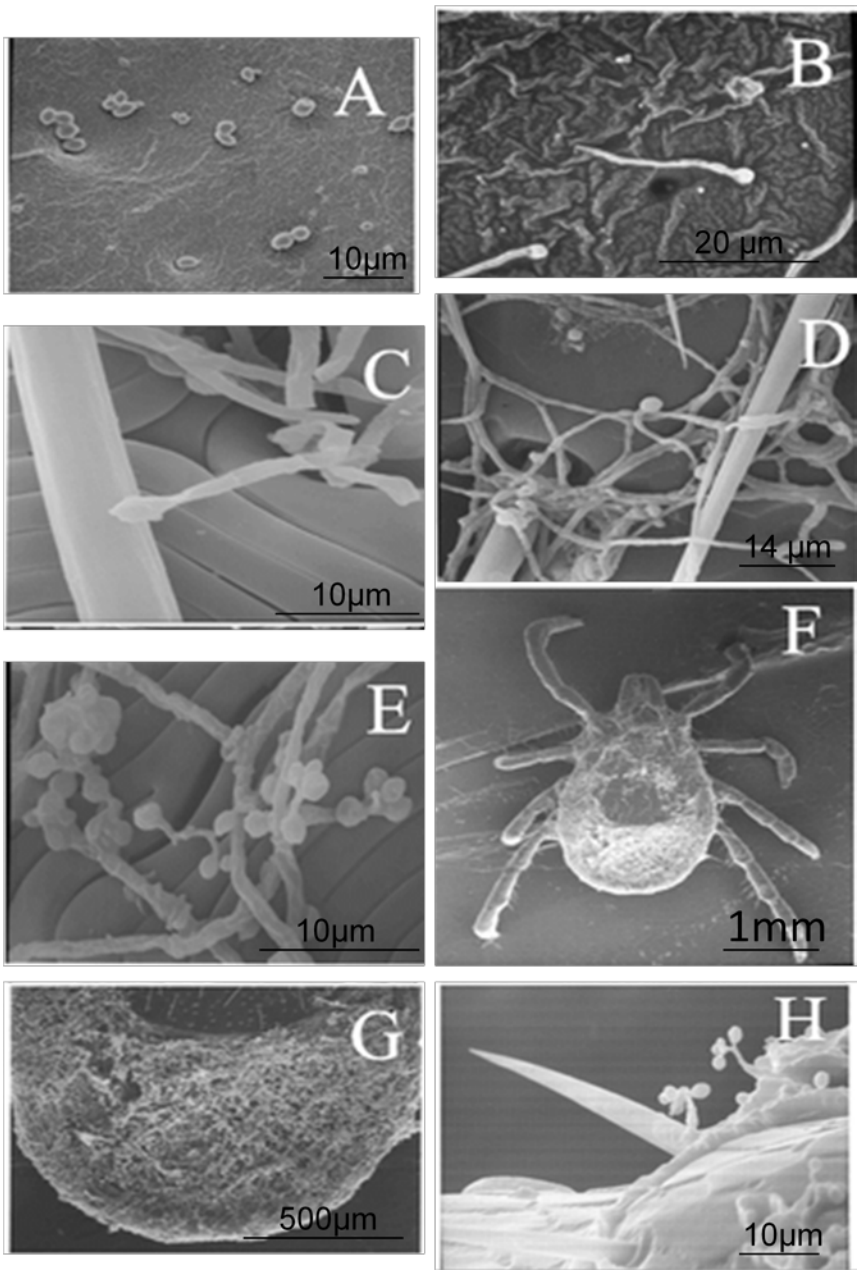


Figure 1-9. Electron micrographs of the *B. bassiana* conidia-mediated infection process. Conidia bound to tick cuticle, 1 hour postinfection (A, *R. sanguineus*, 1,400×). Conidial germination, 18–24 hour postinfection (B, *I. scapularis*, 1,300×; C, *D. variaibilis*, 3,600×). Proliferation and mycelial formation across the tick cuticle surface 7–14 dats postinfection (D, *I. scapularis*, 2,000×; E, *D. variabilis*, 3,400×). Tick cadaver 21–28 days postinfection, illustrating extensive growth particularly in the posterior region of the tick (F, *R. sanguineus*, 15×; G, *R. sanguineus*, 30×). Conidiogenesis, outgrowth of conidia from tick cadaver 28 dats postinfection (H., *D. variabilis*, 1,800×).

Table 1-4. Effect of inoculum composition on *B. bassiana*-induced mortality against *R. sanguineus*, *D. variabilis*, and *I. scapularis*

Treatment	*D. variabilis*	Mortality[a] (%) *R sanguineus*	*I.scapularis*
Sab broth[a]			
7d post infection	0	2±2	0
14d	3±1	2±2	2±2
21d	5±3	6±3	6±2
28d	5±3	7±3	7±3
10^7 conidia/ml in Sab broth[b]			
7d post infection	0	3±2	ND
14d	1±5	9±3	ND
21d	9±2	15±3	ND
28d	11±2	28±6	ND
10^8 conidia/ml in Sab broth[c]			
7d post infection	0	4±2	2±1
14d	7±3	37±8	15±4
21d	15±5	44±10	40±10
28d	19±6	60±15	45±10
Washed 10^7 blastospores/ml			
7d post infection	0	4±2	2±1
14d	4±1	7±3	10±3
21d	8±3	12±5	20±5
28d	11±5	39±7	33±8
Unwashed 10^7 blastospores/ml[d]			
7d post infection	6±3	4±2	6±2
14d	29±8*	32±3	15±3
21d	43±10*	50±11	44±5
28d	60±15*	65±12	53±8
Washed 10^7 blastospores in spent broth[e]			
7d post infection	5±2	3±1	10±5
14d	25±4*	26±4	22±7
21d	40±10*	43±4	39±10
28d	55±11*	58±10	55±12

ND, not determined

* Indicates significant ($P<0.001$) between test treatment and inoculations with washed blastospores, 10^8 condia per milliliter ±broth and controls (Tween20). [a]Ticks were inoculated with a 1:10 dilution of Sabouraud (Sab) broth. Mortality of ticks treated with sterile dH_20 was < 5% throughout the time course of the experiments. [b]Condiia were resuspended in sterile dH20 supplemented 1:10 with Sabouraud broth. [c]Cell culture was filtered through glass wool to remove mycelia clumps and used directly as inoculum. [d]Cells were filtered and harvested as described in Materials and Methods, washed once with sterile dH_2O and resuspended in filter sterilized spent media (0.22 µm filter). [e]Ticks were inoculated with sterile 1:10 dilution of Sabouraud media.

Table 1-5. Acaracide activity towards adult *A. americanum*, oxalic acid concentration, and pH of cell-free *B. bassiana* culture supernatants.

Spent growth media[a] (6 day culture supernatants)	% mortality[b]	[Oxalic acid][c]	pH[d]
	A.americanum, 14d	mM	spent culture
SD	20±8	18	4.5
SD + 1% yeast extract	48±15	23	4.2
PD	12±4	12	5.5
CzD	6±4	0.5	6.8

[a]Fungal cells were removed from liquid cultures by centrifugation. The resultant supernatants were filtered through 0.22 mm filters and stored until use. [b]In all instances, <5% tick mortality was observed in control experiments using fresh media or sterile dH_2O over the same course of the experiment. [c]No oxalic acid was detected in fresh media. [d]Initial pH values for the media were 5.6 (SD, SD + YE, and PD) and 7.3 (CzD).

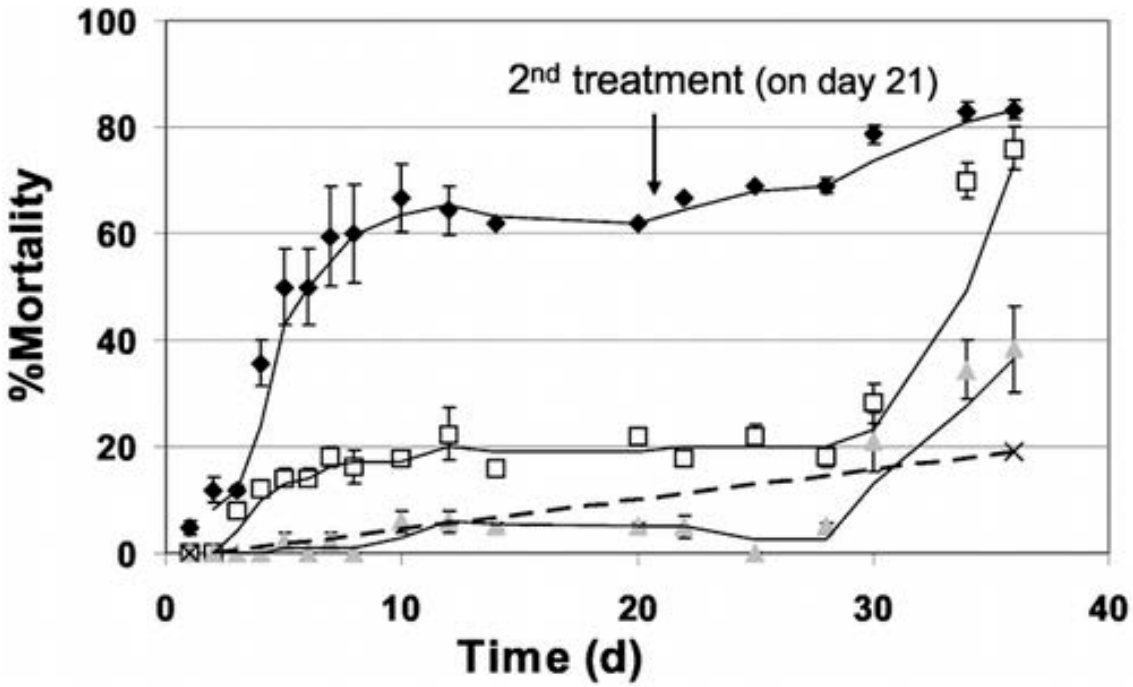


Figure 1-10. Oxalic acid-induced mortality in adult *A*. *americanum* ticks. Ticks were treated with solutions of 50 mM oxalic acid, pH 4.0 (♦); 20 mM oxalic acid (□), pH 4.0; and 50 mM oxalic acid, pH 7.0 (>); all other conditions tested including 1, 5, 10, 20, and 50 mM citrate, pH 4.0 and 7.0, 1, 5, 10, 20, and 50 mM formate, pH 4.0 and 7.0, 1, 5 and 10 mM oxalate, pH 4.0, and 1, 5, 10, and 20 mM oxalic acid, pH 7.0 (dashed lines between the X marks). Values given are means of three experiments ± SE.

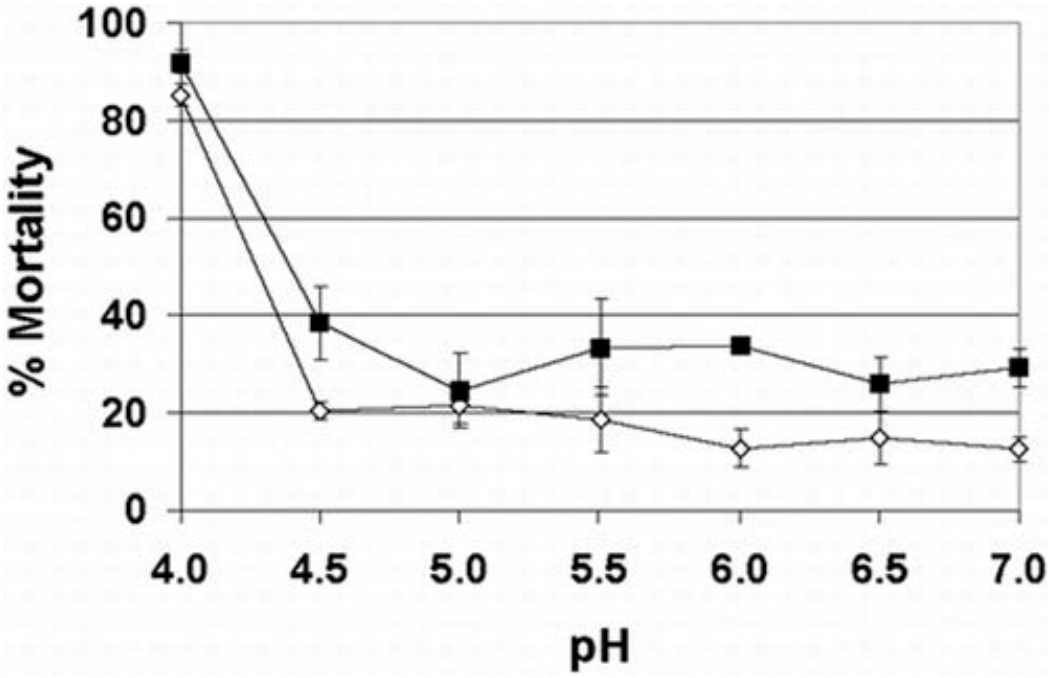


Figure 1-11. pH dependence of oxalic acid-induced mortality in adult *A*. *americanum* ticks. Mortality of adult *A*. *americanum* 14 d after treatment with 50 mM solutions of oxalic acid at the indicated pH values (), and mortality 14 days later, after a second treatment on day 14 (28-days total, ▪). Values given are means of at least three experiments ± SE.

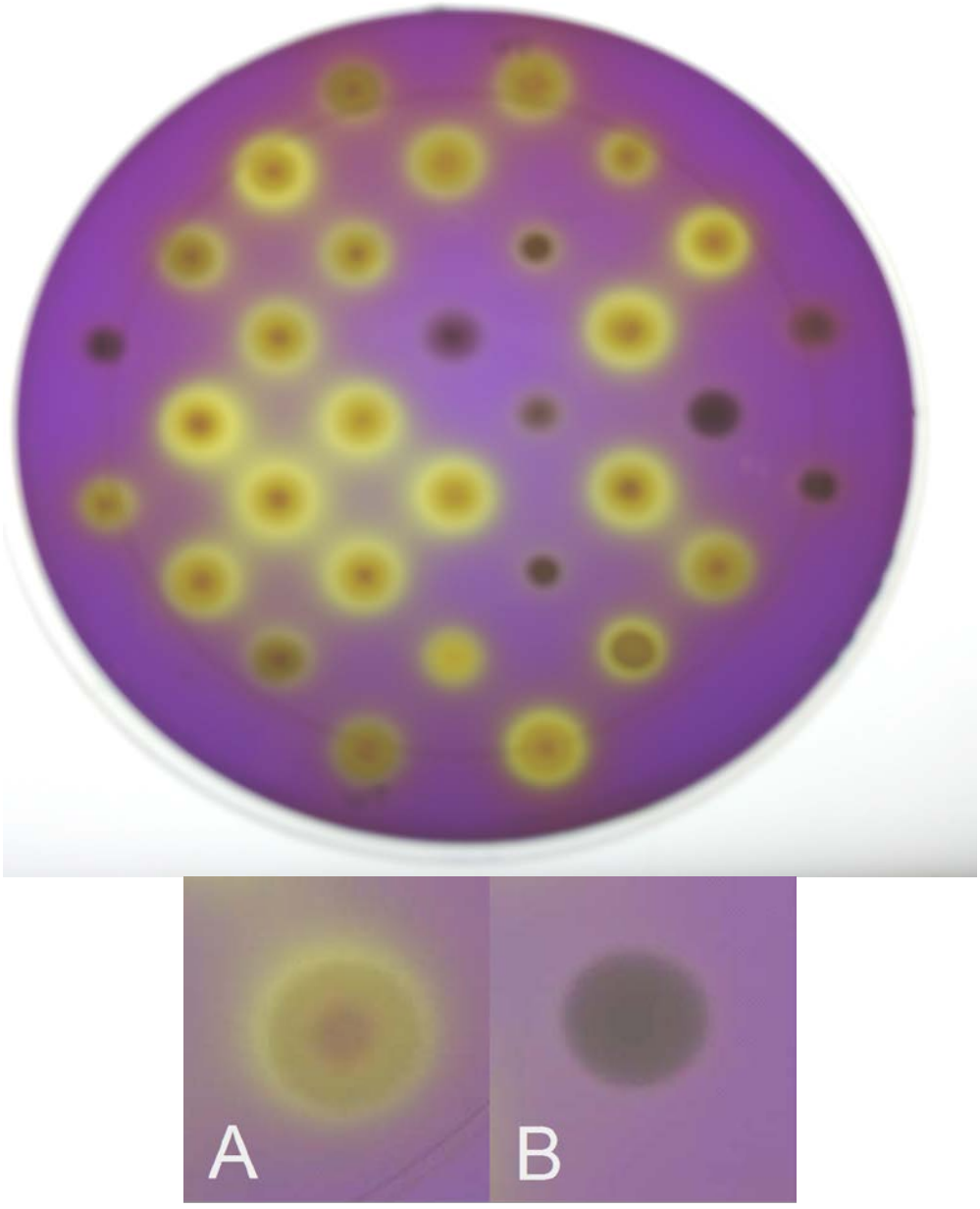


Figure 1-12. Mutant screens of oxalic acid nonproducers. EMS-treated conidia were plated on SDY media supplemented with bromcresol purple. A) Oxalic acid producer B) Oxalic acid nonproducer.

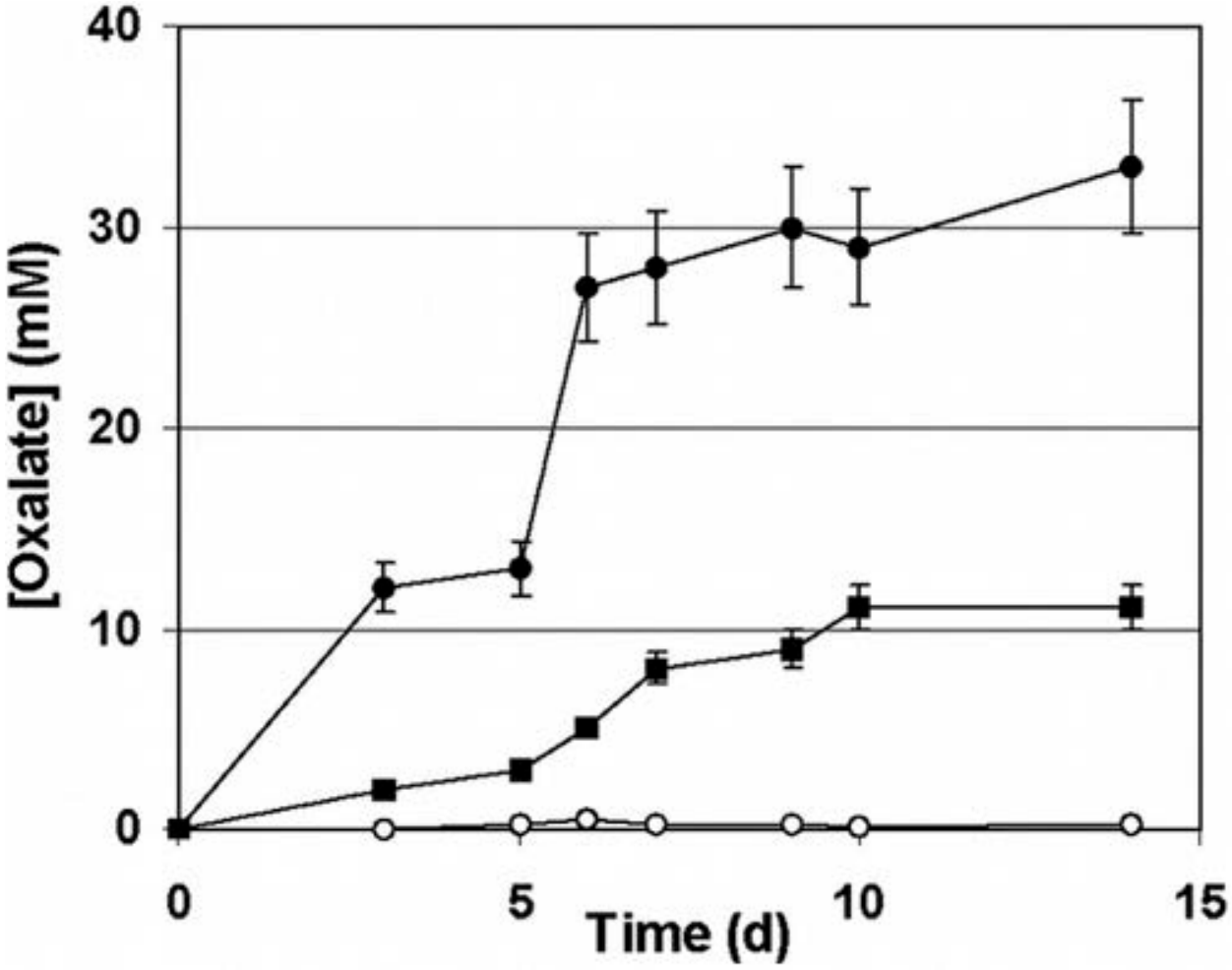


Figure 1-13. Concentration of oxalic acid secreted into the medium during growth in SDY broth, wild-type *B*. *bassiana* (•), mutants A + 15 and A + 16 (○), and mutant A+ 17 (▪).

CHAPTER 2
SURFACE CHARACTERISTICS AND HYDROPHOBINS OF *Beauveria bassiana*

Introduction

Cell surface attachment is a required step in establishing mycosis during host-pathogen interactions and can be considered a primary process mediating fungal pathogenicity (Boucias *et al.*, 1988). The cell surface properties of many fungi form the basis for the initial step mediating interactions with arthropod hosts (Boucias *et al.*, 1988; Hajek & Eastburn, 2003; Hazen *et al.*, 1990). These surface properties are often mediated by fungal spore coat proteins, including a class of proteins known as hydrophobins, which play an important role in attachment (Holder & Keyhani, 2005; Talbot *et al.*, 1996; Tucker & Talbot, 2001; Wessels, 1997; Wosten *et al.*, 1993). *B. bassiana* produces several mono-nucleated cell types that include aerial conidia, blastospores, and submerged conidia. These cell types display distinct cell wall characteristics, as exemplified by differences in hydrophobicity and lectin-binding properties (Boucias *et al.*, 1988; Boucias & Pendland, 1991; Hegedus *et al.*, 1992; Jeffs *et al.*, 1999). The hydrophobic surface attachment property of *B. bassiana* aerial conidia is attributed to a proteinacious spore coat called the rodlet layer, which consist of hydrophobin proteins (Holder *et al.*, 2007).

Hydrophobins are a group of small (8-14kDa) secreted amphipathic proteins produced by filamentous fungi. Gene expression studies of two class I hydrophobin genes were performed to assess the relative abundance of these transcripts during the life cycle of the fungus including within the three cell types described above, namely, aerial conidia, blastospores, and submerged conidia as well as during mycelia growth. The *hyd1* gene was shown to be constitutively expressed during all growth periods and

cell types whereas the *hyd2* gene was primarily expressed in growing mycelia. Data revealed the differential regulation of these genes, implying potential differing developmental roles for their protein products.

Hydrophobins can alter the hydrophobicity of glass, HOPG, Teflon, polystyrene, and other commonly used materials (Linder *et al.*, 2002; Lumsdon *et al.*, 2005; vanderVegt *et al.*, 1996; Wosten *et al.*, 1993; Wosten *et al.*, 1994). On the basis of these observations, hydrophobins have been suggested for use in a variety of biotechnical surface modification (Janssen *et al.*, 2002; Scholtmeijer *et al.*, 2002). However, due to the difficulty in the production and purification of homologous class I protein, it has been difficult to facilitate biotechnical surface modifications (Scholtmeijer *et al.*, 2001). In response to these limitations, a modified *hyd2* gene was successfully cloned and its protein product expressed in an *Escherichia coli* host using the pTWIN1 vector. The protein was purified from the recombinant *E. coli* host and characterized. The recombinant nHyd2 protein was shown to self-assemble into a 2-dimensional array on a glass surface and change the wetability of the surface. Also, the purified protein restored the phenotype in trans-complementation experiments of a *B. bassiana* Δ*hyd2* targeted gene knockout in a charge dependent manner.

Literature Review

Surface Characteristics of Entomopathogenic Fungi

Insect cuticles represent a significant barrier to microbial pathogens with attachment and subsequent germination of infective fungal propagules essential to establishing mycosis (Boucias & Pendland, 1991; Fargues, 1984; Pendland *et al.*, 1993). Infection by *B. bassiana* depends on attachment of the spore to the host surface

and is a prerequisite for virulence specific development such as germination, appressoria formation, and secretion of cuticle degrading enzymes.

Current models that consider the interactions that mediate surface adhesion between fungal cells and substrata involve a consolidation of adhesion by receptor-ligand interactions and non-specific interactions. Nonspecific interactions work by the action of van der Waals forces, surface hydrophobicity, and electrostatic surface charge (Boucias *et al.*, 1988; Smith *et al.*, 1998). These forces are reversible and are dependent on environmental conditions and the nature of the substrata as well as that of the infecting spore (Boucias *et al.*, 1988; Dunlap *et al.*, 2005; Holder & Keyhani, 2005; Smith *et al.*, 1998). Cell surface hydrophobicity has been demonstrated to be an important factor in the pathogenicity of several fungal pathogens including *Candida albicans*, an important human opportunistic fungal pathogen, *Entomophage maimaiga*, a lepidopteran specific pathogen, *B. bassiana* and *M. anisopliae* which are broad host entomopathogens (Boucias *et al.*, 1988; Hajek & Eastburn, 2003; Holder & Keyhani, 2005; Smith *et al.*, 1998). The level of surface hydrophobicity comes from the relationship between hydrophobic surface molecules and other hydrophilic regions in the cell wall (Smith *et al.*, 1998). In view of the role of small surface proteins called hydrophobins, this relationship is especially important. Hydrophobins are usually attributed to the strong surface hydrophobicity of aerial conidia (Bidochka *et al.*, 1995b; Ebbole, 1997b; Holder & Keyhani, 2005; Holder *et al.*, 2007; Lugones *et al.*, 1996b; Paris *et al.*, 2003; Talbot *et al.*, 1996; Wessels, 1997; Wosten & de Vocht, 2000). However, there are a few instances of non-hydrophobic proteins being associated with cell surface hydrophobicity (Lugones *et al.*, 2004). When the hydrophobin SC3 from *S.*

commune was deleted there was a decreased ability, but not completely, to attach to hydrophobic surfaces which suggested other factors may be involved in the hydrophobic surface attachment. Lugones et.al demonstrated that a non-hydrophobic protein, SC15, was responsible for limited attachment in the absence of the SC3 hydrophobin (Lugones *et al.*, 2004).

Specific binding proteins that are important in attachment include cell wall proteins, specific ligand-receptor moieties, and adhesins (Boucias *et al.*, 1988; Doss *et al.*, 1993; Lacroix & Spanu, 2009; Pendland & Boucias, 1991; Prados-Rosales *et al.*, 2009; Sharifmoghadam & Valdivieso, 2008; Szilvay *et al.*, 2007; Tucker & Talbot, 2001). Cell surface polysaccharides may participate in both non-specific and specific interactions, i.e. via cell surface charge masking or recognition by lectins respectively. The specific interactions are considered non-reversible due to the physiochemical forces involved. The specific surface carbohydrates include glucose, galactose, mannose N-acetylglucoseamine and fucose which are implicated in cell adhesion, stress survival and immune evasion (Wanchoo *et al.*, 2009). The MAD1 and MAD2 adhesins of the entomopathogenic fungus *M. anisopliae* has been shown to mediate specific adhesion to insect and plant surfaces respectively (Wang & St Leger, 2007). Targeted gene disruption of these adhesins resulted in a 90% reduction in adherence insect and plant surfaces. The disruption in MAD1 and MAD2 also showed a decrease in spore germination and an altered morphology accompanied by a down regulation of genes involved in the cytoskeleton and cell cycle (Wang & St Leger, 2007). This further demonstrates the crucial role of attachment and adherence forces in colonization during host pathogen interactions.

Conidia, blastospores, and submerged conidia produced by *B. bassiana* can be easily distinguished by size, shape, and surface characteristics (Holder & Keyhani, 2005; Holder *et al.*, 2007). Studies on the adhesion properties of the *B. bassiana* cell types revealed that aerial conidia are able to adhere rapidly to both hydrophobic and hydrophilic surfaces; blastospores display a high degree of binding to hydrophilic substrates; and submerged conidia have a broad but weak binding capability to hydrophobic, weakly polar, and hydrophilic substrates (Holder & Keyhani, 2005). The strong hydrophobic surface attachment property of aerial conidia is attributed to a proteinacious sporecoat called the rodlet layer (Boucias *et al.*, 1988). This rodlet layer is an amyloid-like filament structure of mosaic bundles approximately 10-20 nm in length found on the surface of the spores of most filamentous fungi. The presence of a rodlet layer seems to increase the relative hydrophobicity whereas the absence of this structure results in the more hydrophilic spores. The *B. bassiana* rodlet layer has been extracted and determined to be composed of at least one protein known as a hydrophobin (Holder *et al.*, 2007).

Hydrophobins

Hydrophobins are a family of low molecular weight amphipathic proteins unique to the Fungal Kingdom. Although there is little sequence conservation, they are commonly characterized by their hydrophobicity plots and the presence of eight spatially conserved cysteine residues (de Vocht *et al.*, 2000; Kershaw *et al.*, 2005; Kwan *et al.*, 2008). They undergo spontaneous polymerization allowing them to assemble at the fungal cell wall and at hydrophobic/hydrophilic or liquid/air interfaces (Fan *et al.*, 2006; Whiteford *et al.*, 2004). Once self-assembled, they form monolayers that are highly insoluble. For example, the extraction and solubilization process for some

hydrophobins requires high concentrations of triflouroacetic acid or formic acid (Linder *et al.*, 2002; Zhao *et al.*, 2007). Structural analysis of several hydrophobins has lead to the hypothesis that these proteins exist in three conformational states; monomeric, α-helical, and β-sheet (de Vocht *et al.*, 1998; Fan *et al.*, 2006; Kwan *et al.*, 2006). In solution the protein exists in the monomeric state. Upon binding to a hydrophobic solid substrata, the hydrophobins will shift to a α-helical state, where as self-assembly at the liquid-air interface is dominated by the β-sheet conformation (de Vocht *et al.*, 2000). Studies on the self-assembled form of EAS, a class I hydrophobin, revealed that it is comprised of a β–barrel core with two disordered regions and another two stranded β-sheet region. Furthermore all the charged amino acids are localized on the same interfacial surface, thereby conferring its amphipathic nature. Figure 2-1 shows a possible representation of how these hydrophobin monomers are stacked at the water/air interface or hydrophobic/hydrophilic interfaces. One side of the monolayer is highly hydrophobic while the other is highly hydrophilic allowing it, once polymerized, to form a sheet at the water/air or hydrophobic/hydrophilic interfaces.

Hydrophobins can be further categorized into two classes based upon the distribution of hydrophilic/hydrophobic residues and solubility characteristics. Class I hydrophobins are very stable under denaturing conditions such as 1% SDS, 60% ethanol and form a protein monolayer that appears in form to look like small rodlets, and that can only be dissolved in strong acids such as triflouroacetic acid (TFA). Class II hydrophobins are typically smaller in molecular weight than their class I counterparts and also form self-assembled monolayers, but these are much less stable and can be

solubilized by detergents and alcohols such as 1% SDS and 60% ethanol (Kwan *et al.*, 2006; Wosten & de Vocht, 2000).

There is a high degree of sequence variation among hydrophobins with little to no primary amino acid conservation aside from the cysteines. However, there is an amphipathic secondary core structure found in all hydrophobins examined to date that results in conserved 3-dimensional structural morphology and is responsible for self assembly property. Hydrophobins typically contain a secretion signal that targets the protein as extracellular. This secretion signal is cleaved during maturation. There are also eight spatially conserved cysteine residues which form four intramolecular disulfide bonds (de Vocht *et al.*, 2000; Kwan *et al.*, 2008). These cysteine residues are important for proper stability, secretion, and cell wall localization of the protein (Kershaw *et al.*, 2005). Disruption of some disulfide bridges in Mpg1 of *M. grisea* revealed that these cysteine residues are essential for rodlet layer formation. Disruption of the Cys3-Cys4 disulfide bridge did not inhibit self assembly but resulted in proteins that did not localize to the cell surface. An important conclusion from these studies was that the β-barrel core structure is stabilized by the disulfide linkages and appears to be necessary for the monomers to localize on the cell surface (Kershaw *et al.*, 2005). Based upon structural modeling, Figure 2-2 shows the presumed disulfide linkages of the two class I hydrophobins, Hyd1 and Hyd2, of *B. bassiana*. This disulfide linkage profile has been demonstrated for class I and class II hydrophobins, EAS from *Neurospora crassa*, RodA from *Aspergillus nidulans*, HFBI and HGBII from *Trichoderma reesei*, SC3 from *Schizophyllum commune*, and MPG1 from *Magnaporthe grisae* (de Vocht *et al.*, 2000; Kwan *et al.*, 2006).

Thus far there have been over 50 hydrophobin genes isolated from fungal species from ascomycetes to basidiomycetes. Most of the genes have been identified from either mRNAs or insoluble surface proteins. The regulation of these genes is controlled by several factors which include the following; parental mating seen in SC3, SC4, and SC6 of *S. commune*, circadian patterning and nutrient starvation regulation of EAS in *N. crasssa*, and nutrient regulated expression of MPGI, HFBI and HFBII from *M. grisae* and *T. reesei* (Bellpedersen *et al.*, 1992; Lau & Hamer, 1996; Talbot *et al.*, 1993; Wessels, 1997). Differential expression of *Le.hyd1* and *Le.hyd2* hydrophobins from *Lentinula edodes* revealed the developmental regulation and specific expression during the fruiting process (Ng *et al.*, 2000). Although the role of hydrophobins in some cell processes remains unclear; they have been implicated in a variety of developmental processes including pathogenesis, fruit body formation, and sporulation, (Beckerman & Ebbole, 1996b; Bell-Pedersen *et al.*, 1992; Ebbole, 1997a; Girardin *et al.*, 1999a; Kazmierczak *et al.*, 2005b; Kershaw & Talbot, 1998; Lugones *et al.*, 1996a; Nishizawa *et al.*, 2002).

Due to their unique biophysical properties such as self-assembly at hydrophobic/hydrophilic interfaces and stability under normally denaturing conditions, hydrophobins have been denoted as “proteins with potential” for a wide range of biotechnological applications (Hektor & Scholtmeijer, 2005). These applications include hydrophobins as agents capable of surface modification, acting as antifoulants, as components of biosensors, and as a scaffolding for 3D tissue engineering (Hektor & Scholtmeijer, 2005). Hydrophobins are currently being studied for their ability to alter hydrophobic surfaces such as Teflon and hydrophilic surfaces like mica (Corvis *et al.*,

2005; Janssen *et al.*, 2002; Qin *et al.*, 2007). In these studies, the native hydrophobin which is directly harvested from fungal cells have been coated onto mica and teflon. Once coated, there is a significant difference in the surface hydrophobicity. When class I hydrophobin HGFI from *Grifola frondosa* was coated on to a hydrophobic surface such as teflon there was a decrease in water contact angle from 123.0^{o} to 104.4^{o}, when coated on a mildly hydrophilic surface (silanized glass) there was a decrease from 86.6^{o} to 51.9^{o}, and when coated onto a strongly hydrophilic surface (freshly cleaved mica) an increase of 0.3^{o} to 17.9^{o} was shown (Hou *et al.*, 2009; Yu *et al.*, 2008). Similar results have also been shown with class I hydrophobin EAS and SC3 from *Neurospora crassa* and *Schizophyllum commune* respectively (Askolin *et al.*, 2006; Kwan *et al.*, 2008). This ability to alter the wettability of a surface is a key component in the design and fabrication of biotechnological surfaces. One such biotechnical application of hydrophobins has been in the fabrication process of silicon micromachining. A class I hydrophobin from *Pleurotus ostreatus* was coated onto a silicon or silicon oxide sample. The samples were then KOH etched as part of the silicon micromachining process. It was demonstrated that the hydrophobin provided an effective shielding against exposure to KOH (De Stefano *et al.*, 2007). There is also work using modified hydrophobins to promote fibroblast growth. The hydrophobin SC3 from *Schizophyllum commune* was genetically fused to the RGD adhesion peptide from fibronectin, resulting in a protein that did not alter the self-assembly properties for the SC3 hydrophobin, but did increase binding of fibroblast to a teflon surface (Janssen *et al.*, 2002; Scholtmeijer *et al.*, 2001). Also the class II HFBI from *Trichoderma reesei* was used as a immobilization platform for constructing an amperometric glucose biosensor which

demonstrated that the hydrophobin had an ability to provide a immobilization matrix with biocompatibility and electroactivity (Zhao *et al.*, 2007). More recently, the surface hydrophobin RodA from *Aspergillus fumigatus* has been shown to hide conidial spores from specific immune components. Specifically the hydrophobin was unable to cause stimulation of lymphocytes and CD4+ T-cells, or cause the maturation of dendritic cells (Aimanianda *et al.*, 2009). This cell specific non-immunogenicity may become an attractive quality for targeted biomedical surface modification. Overall, the unique biophysical and biochemical properties of hydrophobins, such as self-assembly, stability under normally denaturing condition, and their amphipathicity, make them interesting proteins for study.

Using a phage display cDNA library two *B. bassiana* hydrophobins were isolated. These hydrophobins contained the hallmarks of hydrophobins such as low molecular weight, secretion signal, and the presence of eight spatially conserved cysteine residues. These class I hydrophobins genes were termed *hyd*1 and *hyd*2 (Cho *et al.*, 2007a). The Hyd2 protein has been shown to be a part of the *B. bassiana* spore coat and Hyd1 was thought to be either secreted into liquid cultures, to lower water surface tension allowing fungal structures to grow into the air, or to condition surfaces allowing the fungus to grow. The addition of a pH induced self cleavable intein fusion partner and a step wise refolding of the class I hydrophobin recombinant nHyd2 from inclusion bodies results in active protein that is self-assembling and can alter the surface hydrophobicity of glass. It will be shown that both *B. bassiana* hydrophobins Hyd1 and Hyd2 are responsible for the formation of the rodlet layer through targeted gene knockouts (Δ*hyd1* and Δ*hyd2*). Also, a novel trans-complementation system using

recombinant nHyd2 hydrophobin and Δ*hyd*2 conidia will be used to demonstrate a charge dependent self-assembly of nHyd2 on the cell surface.

Materials and Methods

Cultivation of Microorganisms and Chemical Reagents

Beauveria bassiana (ATCC 90517) aerial conidia were grown on potato dextrose agar (PDA) or sabouraud dextrose + 0.5% yeast extract on agar plates (SDAY) containing 5ug/ml Trimethoprim, a broad spectrum antibiotic to reduce bacterial contamination. Agar plates were incubated at 26^{o}C for 10-12 days and aerial conidia were harvested by flooding the plate with sterile d$H_2$0 containing 0.01% Tween20. Conidial suspensions were filtered through glass wool and final concentration determined by direct count using a haemocytometer. Liquid broth cultures (blastorspores) were inoculated (1:20) with conidia harvested from plates to a final concentration of 0.5-5 x 10^5 conidia/ml. Cultures were grown for 3-4 days at 26^{o}C with aeration. Cultures were filtered through glass wool or Miracloth (Calbiochem Corp.) to remove mycelia, and the concentration of blastospores was determined by direct count. Filtered cell suspensions were harvested by centrifugation (10,000 X g, 15 min, and 4^{o}C), washed two times with sterile dH_2O + 0.02% Tween20, and resuspended to a concentration of 10^8 spores/ml. Submerged conidia were produced in TKI liquid media (50.0 g Fructose, 10.0 g KNO_3, 5.0 g KH_2PO_4, 2.0 g $MgSO_4$*7H_2O, 50 mg $CaCl_2$, 50.0 mg Yeast extract in 1L of $H_2$0) with aeration for 3-4 days at 26^{o}C. Submerged conidia were filtered though Miracloth and the concentration was determined by direct count using a haemocytometer.

Microbial Adhesion to Hydrocarbons (MATH) Assay

Cell surface hydrophobicity was determined essentially as described by Smith et al. 1998. Briefly, aerial conidia, blastospores and submerged conidia were washed into PUM buffer (per liter: 22.2 g K_2HPO_4, 1.8 g urea, 0.2 g $MgSO_4$*$7H_20$, final pH 7.1). Fungal cell suspensions were adjusted to OD_{470} 1.4 and dispensed (3 ml) into acid-washed glass tubes (12X75 mm). Hexadecane (300 µl) was then added to each tube and the tubes were vortexed three times for 30 sec. The vortexed tubes were allowed to stand at room temperature for 15 min before the hexadecane phase was carefully removed and discarded. Tubes were then cooled to 5^{o}C and any residual solidified hexadecane removed. The tubes were then returned to room temperature and the A_{470} of the resultant cell suspensions was determined. The hydrophobic index was calculated using the following equation:

$(A_{470,control} - A_{470,hexadecane\ treated})/ A_{470,control}$

Hydrophobic-Interactions Chromatography (HIC) Assay

Fungal cells (1 ml of 1-2 x 10^7cells/ml) washed in PUM buffer were loaded onto 1 ml columns containing either phenyl-sepharose or unmodified (CL4B) sepharose (Sigma) pre-equilibrated in PUM buffer. Columns were subsequently washed in PUM buffer (4 ml) and the number of fungal cells recovered in the eluate was determined using a haemocytometer. The hydrophobicity incex (HI) was calculated using the following equation: [(percentage cells eluted from unmodified sepharose) – (percentage cells eluted from phenyl-sepharose)]/(percentage cells eluted from unmodified sepharose).

RNA Extraction

Total RNA was extracted from *B. bassiana* cells using either RNAwiz or TRI Reagent (Ambion) according to the manufacturer's recommendations, including the high-salt precipitation step for removal of proteoglycans and polysaccharides. Culture conditions for RNA extraction were as follows: *B. bassiana* was grown on PD agar (PDA) or on Sabouraud dextrose + 1% yeast extract either on agar plates (SDAY) or in liquid broth (SDY). Plates were incubated at 26^{o}C for 10-12 days and aerial conidia were harvested by flooding the plate with sterile dH_2O. Conidial suspensions were filtered through Miracloth and final spore concentrations were determined by direct count using a haemocytometer. Liquid broth cultures were inoculated (1:50, v/v) with conidia harvested from plates to a final concentration of 0.5-5 X 10^5 conidia/ml. Blastospore cultures were grown for 3-4 days at 26^{o}C with aeration. Cultures were filtered through glass wool or Mira cloth to remove mycelia, and the concentration of blastospores was determined by direct counting. Submerged conidia were isolated from TKI broth (per Liter: 50.0 g fructose, 10.0 g KNO_3, 5.0 g KH_2PO_4, 2.0 g $MgSO_4$*$7H_20$, 50.0 mg $CaCl_2$, 50.0 mg yeast extract), as previously described (Cho et al., 2006). A time course of growing mycelia was prepared by growing *B. bassiana* on PDA. At the desired time points (3, 5, 10, 18, and 28 days), conidia were removed by washing the plates by flooding two to three times with dH_2O, and the mycelium was obtained by lightly scraping off the fungal biomass from the resultant agar plates. Mycelia were examined by light microscopy for the presence of conidia, and samples containing less than 1% conidia were used for further experimentation. Chitin and insect cuticle liquid broth cultures (50-100 ml), consisting of 1:4 diluted Sabouraud dextrose broth supplemented with 1% (wt/v) chitin (extracted from crabshells), 1% powdered,

sterilized *Manduca sexta* cuticle (kind gift of D. Boucias, Dept. of Entomology and Nematology, University of Florida), or 1% chitin + 1% *M. sexta* cuticle, were inoculated with conidia harvested from plates to a final concentration 0.5-5 X10^5 conidia/ml and were grown for 3 days at 26^{o}C, flash frozen in liquid nitrogen, and stored at -70^{o}C.

Semi-quantitative Reverse Transcriptase PCR Analysis

Total RNA isolated as described above was precipitated once with LiCl before being DNase-treated using the DNA-free reagent (Ambion). RNA samples were then treated with SUPERase-In (RNase inhibitor, Ambion) and stored at -70^{o}C until use. Total RNA concentration was quantified for each sample preparation using the Ribogreen RNA quantification kit (Molecular Proves). cDNA for each sample was synthesized using 1.0 g of total RNA plus Superscript III reverst transcriptase with oligo dT^{18} priming, following the manufacturer's protocol (Invitrogen). PCR reactions were as follows: 1.0 µl of a twofold dilution of the cDNA sample was PCR-amplified using 6.0 µl DNA polymerase Mastermix (Eppendorf), 0.3 µl of 0.01 mM of each primer, and dH_2O to a final volume of 17.0 µl. Amplifications of portions of the *B.bassiana actin* and *β-tubilin* genes (See Table 2-1) were performed as controls during all reactions, and were used as internal standards to normalize the expression levels of the *hyd1* and *hyd2* genes in the various RNA samples. The relative intensity of the bands was determined after densitometric scanning using Adobe Photoshop. Primer sets used for the amplification of *hyd1*, *hyd2*, *actin*, and *β-tubulin* are presented in table 2-1. In all experiments, controls containing no template or no enzyme were performed. Each PCR reaction was performed twice with duplicate biological samples and cDNA preparations for each sample.

Isolation and Construction of *nHyd2* Gene into the pTWIN1 Expression Vector

Restriction enzymes were obtained from NEB. *E.coli* competent cells Top 10, BL21 and Rosetta 2 (DE3) were purchased from Invitrogen. All other chemicals and reagents were purchased from Fischer Scientific.

The open reading frame (ORF) corresponding to the *hyd*2 gene without its predicted signal peptide was cloned into the pTWIN1 plasmid of the IMPACT system encoding an N-terminal modified Ssp DnaB self-cleavable intein tag coupled to a chitin binding domain (New England Biolabs Inc).

The *hyd2* gene was isolated from a *B. bassiana* EST library constructed by Dr. Cho as described previously (Cho *et al.*, 2007a). The *hyd2* was first cloned into a pCR TOPO TA cloning vector from Invitrogen. Primers for isolation of the gene were constructed based upon the cDNA sequence of the *hyd2* gene (Table 2-2). The signal peptide of the *hyd2* gene was predicted by SignalP and not included in the vector construct. The primers included restriction sites for 5' *Sap*I and 3' *Pst*I sequences. The PCR product was run on a 1% agarose gel, cut out and isolated using Qiagen's QIAquick Gel Extraction Kit. Restriction digestion of the *hyd2* fragment and the vector expression plasmid,ligation, and transformation into XL-10 Gold (Stragenene) chemically competent E. coli cells were performed using standard protocols. Transformants were then screened for the correct insert by PCR, *Hind*III/*Nde*I digestion, and DNA sequencing. A stock (1 ml) of culture was then stored at -80^{o}C in 7% glycerol for future work.

Expression and Purification

The pTWIN1 plasmid containing the *hyd2* gene was transformed into *E. coli* Rosetta2 gami cells (Invitrogen), which contains the pRARE plasmid encoding for

several rare tRNAs, aga (Arg), agg (Arg), ata (Ile), cta (Leu), gga (Gly), ccc (Pro), cgg (Arg). These rare tRNA's are needed for expression of the nHyd2. The culture was expressed in 1 liter LB supplemented with ampicillin (60 µg/ml) and chloramphenicol (30 µg/ml). Culture was grown to an OD of 0.9 and induced with 1mM IPTG for 3 hours at 37^{o}C with shaking. The culture was then spun down (12,000Xg), resuspended in 100ml lysis buffer (20 mM Tris, 300 mM NaCl, pH 8.5), sonicated using a Sonifier Cell Disruptor model 185 (Heat Systems-Ultrasonics Inc.) for three 45 sec bursts, and spun down again. The resulting pellet was then suspended in 100 ml denaturing buffer (7M guanidine-HCl, 20 mM Tris, 300 mM NaCl, and 10 mM DTT, pH 8.5) for at least 1 hour at 4^{o}C. The unfolded protein was then refolded by stepwise dialysis against decreasing concentrations of urea (Table 2-3) as previously described (Hackenberger *et al.*, 2006). Each step was performed at 4^{o}C for 24 hours. Roughly 1 L dialysis buffer was used for 1 L culture (100 ml of resuspended pellet in denaturing buffer). The refolded Intein-nHyd2 fusion protein was then loaded onto a 10 mL chitin bead column (New England Biolabs). The column was then washed with 15 column volumes of wash buffer (20 mM Tris, 600 mM NaCl, pH 8.5). The pH mediated intein cleavage was initiated by addition 7 column volumes of cleavage buffer (20 mM Tris, 300 mM NaCl, 1 mM DTT, pH 6.5) for 24-40 hours at room temperature. The nHyd2 protein eluate was collected in 7X 1mL fractions of cleavage buffer. Protein concentration was determined by Pierce 660 nm Protein Assay. The purity of the protein was monitored by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) (Fig. 2-10). Aliquots of the protein samples were mixed with 4X lithium dodecyl sulfate (LDS) and run on a 10% bistris NuPAGE gel with MES running buffer along with protein molecular weight standards

(Invitrogen). Protein bands were visualized using SimplyBlue SafeStain (Invitrogen). For identification of the nHyd2 protein product, a protein band ~11kDa was excised from the PAGE gel and subjected to in-gel tryptic digestion before LC-MS/MS analysis (performed by the ICBR Proteomics Core Laboratory at the University of Florida). Results were analyzed using Scaffold Viewer Proteome software v. 2.01.01.

Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM)

Atomic force micrographs were made using a Digital Instruments Multimode SPM atomic force microscope (model MMAFM-2) placed on a marble stone platform with a nitrogen suspension table. Images were taken in contact mode using a Si_3N_4 probe (Digital Instruments, model NP-20, spring constant = 0.12 N m^{-1}). Fungal cells were placed on 1.2 µm pore-size Millipore filters and air-dried for 1-4 hours before examination. Images were collected at 512 samples/lines with a scan rate of 0.96Hz and tip velocity of 30.6 µm s^{-1}. Data from the micrographs were analyzed using Nanoscope SPM v4.42, SPM Image Magic demo v1.10, or WsXM 3.0.

Transmission electron micrographs were made using a Hitachi H7000. A 200 µl drop of 0.18 µg/ml hydrophobin protein was placed on top of a formvar grid. The grid was then incubated at room temperature overnight to allow the droplet to evaporate. The sample was then negatively stained with uranyl acetate. The resulting film was then visualized by ICBR electron microscopy department in order to determine the presence of recombinant nHyd2 protein.

ThT Assay

It has been previously shown that increased agitation of hydrophobin solution promotes self-assembly via conversion of the monomeric form to the two-dimentsional arrays (Kwan *et al.*, 2008). This property allows us to monitor the nHyd2 transition from

the soluble form to the assembled form. ThT is a dye that when bound to stacked β-sheets displays and increase in fluorescence at 485nm. Thus, due to the stacked β-sheet structure of hydrophobins in self-assembled form, ThT can be used to monitor hydrophobin self-assembly. Solutions containing various concentrations of purified nHyd2 and ThT were vortexed for varying intervals and the fluorescence at 485nm was determined. The ThT binding assay was performed essentially as described (Kwan *et al.*, 2008). Briefly, ~150 µg/ml and ~75 µg of *E.coli* produced Hyd2 and ~150 µg/ml of nHyd2 harvested from conidial surface was mixed with 38 µM Thioflavin-T added to wells of a 96 well black fluorescence plate. The plate was then sealed using iCycler iQ Optical Quality Sealing Tape (Bio-Rad). The samples were then vortexed for intervals of 0, 2, 5, 7, and 10 min. The addition of the nHyd2 and ThT was staggered such that all vortexing time points ended simultaneously in order to read all samples using one plate. The sealant was removed and samples read using a SpectraMAX GeminiXS fluorescence spectrophometer (Gemini Devices). Fluorescence spectra were monitored over a wavelength range of 450-600 nm (with slit widths set at 10nm for excitation and emission) with an excitation of 435 nm and a cut off of 455 nm. Results shown are from three separate replicates.

Hyd2 Glass Surface Modification

Glass cover slips were prepared by cleaning the glass coverslips with detergent and copious rinsing with milliQ distilled H_20 followed by an acid wash (1M HCl) for 2 hours after which the coverslips were copiously rinsed with diH_2O. Purified protein, 50 µl droplet of nHyd2 (20 µg/ml) in elution buffer, was placed onto parafilm. The droplet was incubated overnight in a moist environment to allow self-assembly at the water/air interface. The hydrophobin monolayer was then transferred to a prepared glass cover

slip by bringing it in contact with the hydrophobin droplet. The glass cover slip was then washed 2X with diH_2O, 1% SDS, and 60% ethanol to remove any unbound protein. Assembled monolayer was then visualized by AFM and used for water contact angle measurements.

Water Contact Angle Measurements

Water contact angle and surface tension measurements of the self-assembled recombinant nHyd2 protein were carried out at the Particle Engineering Research Center using a Rame-Hart model 500 advanced goniometer with automated drop dispenser, tilting plate, and DropImage Advanced software. Glass slides were either unmodified, or modified with our recombinant nHyd2. The unmodified glass slides were treated with buffer solution (20 mM Tris, 150 mM NaCl, pH 6.5). The modified glass slides were either prepared by drop transfer method, a 100 µl drop of 130 µg/ml nHyd2 was placed on to a parafilm strip and incubated at RT with relative humidity >90% overnight, glass slide was then placed on top of drop to transfer self-assembled hydrophobin monomer onto its surface; or a 50 µl of 130 µg/ml drop was allowed to evaporate overnight on a glass cover slip. Glass slides were washed 3X with dH_2O and 60% ethanol to remove any excess hydrophobin and/or buffer solution. Contact angle measurements were then determined using glycerin and dH_2O. The angle measurements were determined just prior to movement of the water drop. Briefly, a drop of water or glycerin was brought into contact with the unmodified glass slide and glass slide that had been modified with the self-assembled Hyd2 protein. Advancing and receding contact angles (θ_A and θ_R) were then determined. All experiments were done in triplicate and carried out at room temperature with a relative humidity of 50-55% humidity. Contact angles measurements were taken from 4 separate experiments using

15 angle measurements each. The resulting data was processed using ImageJ 1.42q software.

Langmuir Blodgett Isotherms

LB isotherms were generated with the kind help of Hrishi Basi and Dr. S. Talham. Pressure area isotherms were generated using a KSV Mini Langmuir system (KSV Instruments) using ultra pure Milli-Q water at pH 6.0, and T=24^{o}C ±2^{o}C as the subphase. Purified Hyd2 (600 µl, 130 µg/ml in 20 mM Tris-HCl, 150mM NaCl, pH 6.5) was spread along the subphase using a microsyringe; small droplets were spread about over the whole area (1200 cm^{2}) in order to evenly distribute the sample before compression. Surface pressure was monitored with a small weight attached to a sensitive balance and the sample was allowed to stabilize for 1 hour prior to compression. The monolayer was compressed and expanded at a rate of 250 mm^{2}/min.

∆h*yd*1 and ∆h*yd*2 Knockout Generation

Δhyd1 and Δ*hyd2* gene knockouts were generated by Shizhu Zhang. The targeted disruption of *hyd*1 and *hyd*2 was carried using a blastospore mediated transformation system (Ying & Feng, 2006). Briefly, *hyd*1 and *hyd*2 were amplified using sequencial primer pairs H1F (tcagtctaatgtcgtggtggtggc) H1R (ccaatgttttcggaaccattacccactttgct), H2F (gccacacggcaggctctgagaga), and H2R (ccagcagctgtgcggctacgagat) generating a ~4kb fragments. The PCR product was then sub-cloned into TOPO blunt end vector to generate pTOPO-*hyd*1 and pTOPO-*hyd*2. Reverse PCR was used to linearize the vectors using primer pairs H1-KOF (gcatcgtgttgccgttgccg), H1-KOR (tcaaccagcttgtccccatcgac), H2-KOF (gcatcgtgttgccgttgccg), and H2-KOR (tcaaccagcttgtccccatcgac). The linearized vector was then ligated to herbicide resistance gene (bar) cassette amplified from pBARGPE

to form the hyd1 and hyd2 KO vectors. *B. bassiana* competent blastospore generation and subsequent transformation were conducted by established procedures as described by Ying et.al (Ying & Feng, 2006). Identification of the gene disruption mutants was performed by PCR and DNA sequencing (data not shown).

Trans-complementation of Δ*hyd*2

Trans-complementation was carried out on both Δ*hyd*1 and Δ*hyd*2. Δ*hyd1* and Δ*hyd2* conidia were grown on PDA for 14-21 days. Conidia were then harvested using an inoculation loop and the working solution was kept between 10^6 and 10^7 conidial/ml by direct count using a haemocytometer. The conidia were harvested by scraping the culture plate with an inoculation loop. The conidia were then resuspended in a solution of purified nHyd2 (~130 µg/ml) with 20 mM Tris and 150 mM NaCl. A pipette was used to vigorously agitate the mixture to generate bubbles and incubated at room temperature overnight. The liquid was then evaporated in a centri-vap and the cell pellet resuspended in 100 µl dH_20. The cells were then prepared for imaging by AFM by placing a 10 µl drop onto a 1.2 µm pore-size Millipore filters and air-dried for 1-4h. Successful trans-complementation was determined by the presence of organized bundles having an elongated shape and the presence of striations running lengthwise along the bundles. Quantation of trans-complementation was determined by measuring the length and width of the at least 20 striated bundles (5 conidia each) and comparing to the wild-type conidia. pH dependence of trans-complementation was performed at pH ranges of 4.0-10. Δh*yd*2 conidia were harvested at day 14 and 30 from the same plate. Cells were then trans-complemented with purified nHyd2 solution where the pH had been adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 10.0. Results were determined from at least 15 conidia at each pH. Reactions were also performed using Δh*yd*2 conidia that

were autoclaved, treated with 5% gluteraldehyde, or exposed to UV light. For autoclaving, conidia were placed in a 1.5 ml microcentrifuge tube and autoclaved at 137^{o}C for 2 min. The gluteraldehyde fixed conidia were incubated at room temperature with 5% gluteraldehyde overnight. The cells were then centrifuged at 16,000 x g for 5 min. The gulteraldehyde was then removed by decanting and the cells were washed 3 times with sterile distilled H_20 to remove residual gluteraldehyde. The conidia treated with UV light were smeared onto a Petri dish and exposed to a UV germicidal light for 1.5 hrs. After each treatment the cells were trans-complemented with nHyd2 solution as previously described and imaged by AFM.

Results

AFM: Cell Surface Morphology

Detailed surface topological features of live *B. bassiana* cells could be distinguished by Atomic Force Microscopy (AFM) (Fig. 2-4). AFM allows the visualization of live cells without fixation, and was used to provide resolution at the micrometer level of surface features of freshly harvested cells. Fascicle bundles,presumably composed of assembled hydrophobin(s) protein rodlets, were clearly visible on *B. bassiana* aerial conidia (Fig.2-4a, d). Striated filaments could be distinguished within the elongated bundles (Fig.2-4g). In contrast, no bundles or filaments were visible on either blastospores or submerged conidia. The blastospore surface appeared smooth (Fig.2-4b, e), whereas the submerged conidial surface was rough, and a circular ring was apparent on some of the latter cells (Fig.2-4c, f). In several instances, bipolar germination was noted for *B. bassiana* (Fig.2-4h), and this has been reported to correlate with its infectious nature (Talaei-Hassanloui *et al.*, 2006). No fascicles were visible on the germ tubes or hyphae of germinating aerial conidia,

although fascicles appeared to remain on the conidium during germination (Fig.2-4i, j). AFM images of the germinated conidia revealed fascicles throughout the mother cell, and the lack of fascicles on the slopes of the images presented is due to cantilever artifacts at the resolution employed.

Measurement of Cell Surface Hydrophobicity

Two methods were used to assess the cell surface hydrophobicity of the different *B. bassiana* cell types. In the first, a MATH assay in which cells partition between two immiscible solutions (water and hexadecane) was used (Fig.2-5). In this assay, entities with a hydrophobicity index (HI) > 0.7 are considered hydrophobic (HI=no. cells in organic phase/total no. cells). Aerial conidia were clearly hydrophobic and distributed into the organic phase (HI=0.88), whereas blastospores were hydrophilic, predominantly localizing to the aqueous phase (HI=0.4). Interestingly, submerged conidia partitioned to a slightly greater extent into the organic phase rather than the aqueous phase (HI=0.72), with cell surface characteristics apparently on the borderline between hydrophobic and hydrophilic. A second assay, involving HIC, in which the binding of cells to phenyl-Sepharose and unmodified Sepharose is used as an indicator of the hydrophobic nature of particle surfaces, resulted in data that was in close agreement with the results of the MATH assay (Fig.2-5).

Gene Expression Analysis of the *Beauveria bassiana hyd1* and *hyd2* Genes

The expression patterns of the *B. bassiana hyd1* and *hyd2* genes were analyzed by semi-quantitative RT-PCR. Two internal controls, namely β-tubulin and actin, were used to normalize the expression data. Data in which (i) the variation between samples of the total RNA/actin or total RNA/β-tubulin and (ii) the variation between biological samples was less than twofold were used. In addition, a standard curve of actin

concentration determined by PCR against the amount of RNA guantified by Ribogreen was generated. Only data that fell within the linear portion of the standard curve were considered valid.

The relative expression level of the two genes was assessed in RNA pools derived from aerial conidia, blastospores, submerged conidia, growing mycelia, and from blastospores grown on insect cuticle, chitin (the main carbohydrate constituent of insect cuticles), and insect cuticle + chitin (Fig 2-6). The data presented were determined to be within the linear portion of the PCR analysis (25 cycles), with the actin concentration closely correlating to the amount of RNA quantified for each sample under test. Hydrophobin expression was normalized to the quantification of the actin band. *B. bassiana hyd1* expression was detected in all developmental stages and media conditions tested. Hyd1 levels appeared to increase during mycelia growth, with the highest level of expression observed after 28 days of growth on agar plates, although caution should be taken in any interpretation of these results, as they derived from semi-quantitative measurements. On agar plates cells began sporulating (i.e. producing aerial conidia) after ~14 days; however, conidia were washed from the plates of all the mycelia samples before RNA isolation. This was confirmed by direct count using microscopic visualization of the mycelia samples, in which <1% of the contaminating aerial conidia were visible in the mycelia preparations. Thus, the observed *hyd*1 expression appears to be derived from the mycelia. Expression in aerial conidia was similar to that observed in either blastospores or submerged conidia, and was equivalent to that observed in 18 day mycelia. Cells growing in the presence of 1 % chitin, 1 % cuticle, and 1 % chitin+1 % cuticle also showed robust expression of *hyd1*,

similar to levels seen in blastospores and submerged conidia, and 5–10 day mycelia. In contrast to *hyd1*, *hyd2* appeared to be constitutively expressed and at about the same level as actin throughout the growing mycelia stages (3–28 days). Little or no *hyd2* was observed in either blastospores or aerial conidia. Some *hyd2* transcript was detected in submerged conidia, which corresponded to about 5–10 % of the levels seen in the mycelial samples. Intriguingly, *hyd2* was expressed (at approximately the same levels as actin) in fungal cells growing on 1 % chitin or 1 % cuticle, but almost no transcript could be detected when the cells were grown on 1 % chitin+1 % cuticle.

Protein Expression of Recombinant Hyd2

In order to produce the hydrophobin protein in sufficient quantities for further characterization, a method for using an *E. coli* based production and purification system needed to be established. Numerous attempts were made to express the Hyd2 protein in *E. coli*, including constructing fusion partners with 5X His tags, Thioredoxin fusion moiety's, and the V5 epitope. These systems however, did not produce significant amounts of the desired nHyd2 protein, which lead to the use of the IMPACT (pTWIN1) system from NEB. The *hyd2* gene was cloned into the pTWIN1 vector, transformed into Top10 E.coli and confirmed by PCR, *Hind*III/*Nde*I digestion, and sequencing. In this expression system, an *Synechocystis* sp DnaB intein fusion partner was coupled to the N-terminus of the Hyd2 protein (Fig. 2-7). The expression clone lacked the 17 amino acid signal peptide found in the cDNA ORF and the first three amino acids of the putative mature protein was changed from Ala-Pro-His to Gly-Gly-Ala. This change in amino acids was done to optimize the autocatalytic activity of the intein fusion moiety. The pTWIN-Hyd2 plasmid was then transformed into the Rosetta2 (DE3) for expression. The Rosetta2 (DE3) expression cells carry the pRARE plasmid, which codes for the

rare tRNA's not found in *E.coli*, but are needed to produce the hydrophobin protein. Several strategies such as low temperature, reduced expression time, reduced IPTG concentration, 1% glucose, fusion partners, and constitutive expression were used to express the Hyd2 as a soluble protein, however these did not result in appreciable protein yields. Therefore the nHyd2 protein was purified from exclusion bodies. We decided to purify the nHyd2 protein from the inclusion bodies. After solubilization of the inclusion bodies under denaturing conditions, the refolding process, in order to obtain functionally active protein, relied on a stepwise dialysis of decreasing urea concentrations at a basic pH ~8.5 (8 M Urea, 10 mM DTT; 6 M Urea, 1 mM DTT; 4 M Urea, 1 mM DTT; 2 M Urea, 1 mM reduced glutiothine; 0M Urea, 1 mM reduced glutathione). The stepwise dialysis allows for refolding of the protein, while the basic pH ensures that the fusion moiety is not prematurely removed. After expression and refolding the nHyd2 protein extract was loaded onto a chitin bead column, which was extensively washed and the final product was eluted by addition of buffer at pH of 6.0. LDS-PAGE analysis revealed the presence of a ~11kDa protein in the elution fractions (Fig. 2-8). The eluted protein was then analyzed via tryptic digestion followed by MS peptide fingerprinting. Four peptide fragments, LLAAECSPISVNVLLNQLVPIDNK, LTGPSVLSDLDLR, QQSICCGEQK, and TGDICGNGNTMHCCNDESVTNK were obtained. Peptide analysis of these fragments revealed them to correspond exactly to predicted fragments of Hyd2 amino acid sequence. Protein concentrations of the nHyd2 fractions resulted in roughly 150-250 µg/ml for a total protein yield of 5-10 mg/L of initial *E. coli* culture as determined by the Pierce 660nm protein assay.

Thioflavin T Self-Assembly Assay

nHyd2 at 150 µg/ml displayed a fivefold increase from 130 ± 2.5 to 650 ± 37.8 relative fluorescence units (RFU's) in the first 7 minutes which then plateaud (Fig 2-9). At a nHyd2 concentration of 75ug/ml the fluorescence signal increased ~2.5 fold. The hydrophobin, Hyd2, extracted directly from the conidial surface showed a similar assembly pattern (142 ± 5.8 to 680 ± 12.5 RFU's over 7 minutes) as that of the recombinant nHyd2 at similar protein concentrations. Controls showed no significant increase in RFU's over all time periods.

Transmission Electron Microscopy (TEM)

In order to visually confirm the presence of a self-assembled hydrophobins TEM was used. The purified hydrophobin solution was placed on a formvar grid and sent to ICBR for TEM analysis. The resulting micrographs showed the presence of small striated filaments (Fig 2-10). These filaments are 197.1 ± 52.5 nm long and 24 ± 3.5 nm wide. This further indicates that the *E. coli* produced hydrophobins are assembling into a monolayer just like hydrophobins isolated directly from the fungal surfaces.

LB Blodgett Analysis

To investigate the organization of the protein monolayer at the water/air interface, the nHyd2 was subjected to Langmuir Blodgett compression. The surface pressure vs. area isotherm shows a moderately sharp rise in the surface pressure with a collapse point near 30mN/m, with a mean molecular area of ~28 A^2 (Fig 2-11). These results are consistent with the formation of a rigid monolayer and other hydrophobin monolayers formed at the water/air interface (Asakawa *et al.*, 2009; Houmadi *et al.*, 2008; Kisko *et al.*, 2007).

nHyd2 Surface Modification

Self-assembly of nHyd2 onto a hydrophobic surface was visualized using AFM in contact mode. A monolayer of nHyd2 was deposited upon three glass coverslips using the drop transfer method. An area of 500 X 500 nm was scanned in order to clearly visualize the self-assembled monolayer. Figure 2-12 shows the nHyd2 coated onto a piece of glass using the drop surface transfer method. Experiments were also performed in which a drop of hydrophobin solution was allowed to evaporate on the glass slide. The nHyd2 treated glass slide shows a rigid monolayer. The average height of the monolayer domains seems to be between 2-4 nm, which corresponds well to previously reported results (Houmadi *et al.*, 2008). A roughness analysis showed the roughness (root mean square (RMS)) to be approximately 1 nm throughout the entire sample. The monolayer shows a complete homogenous coverage of the surface with the nHyd2 protein.

Water Contact Angle (WCA) Measurements

A thin film monolayer was generated by coating a glass coverslip with nHyd2 solution and used to determine the change in water contact angle of modified versus unmodified glass slides (Table 2-4). Water contact angle is a measurement of surface hydrophobicity. Contact angle is the angle at which a liquid/air interface comes in contact with a solid surface. When glycerin was used as the liquid substrate, the advancing contacting angle ($\theta_{A)}$ for the control was determined to be 88.9^{o} while that of the modified glass slide was 56.8^{o} using drop transfer (dt) and 68.5^{o} using drop evaporation (de). This is a change in WCA of 32^{o} and 20^{o} respectively. The receding contact angle (θ_R) was determined to be 48.3^{o} for control, 17.5^{o} for dt, and 18.5^{o} for de, with changes in WCA of 31^{o} and 30^{o} respectively. When water was used as the liquid

substrate, θ_A was found to be 75.9^o for control, 70.8^o for dt, and 72.9^o for de. θ_R is 44.5^o for control, 15.6^o for dt, and 14.5^o for de. Figure 2-13 shows images of glycerin and water drops, which clearly show a change in the geometry of the drops from control versus modified glass surfaces.

Δ*hyd*1, Δ*hyd*2 Knockouts and Δ*hyd*2 Trans-complementation

Gene knockouts of the h*yd*1 and *hyd*2 genes in *B. bassiana* were constructed in the lab (S. Zhang). The wild type conidia of *Beauveria bassiana* have elongated bundles (L = 94.15 ± 34.5 nm, W = 36.6 ± 9.8 nm; N = 5 cells, 20 bundles each) with striated filaments (3.8 ± 1.7 filaments/bundle) running lengthwise on the surface (Fig. 2-14a). Aerial conidium in which the Hyd2 protein was no longer produced (*Δhyd2*) has a disrupted spore coat in which the clearly defined bundles and rodlet filaments are lost. Instead a disordered cell surface landscape is noticeable (Fig 2-14c). In contrast, the Δ*hyd1* knockout showed a dramatic disruption in the spore coat phenotype. These conidia lost all appearance of both the bundles and the rodlets found on the sporecoat showing a relatively smooth surface (Fig 2-14d). The *Δhyd1Δhyd2* double mutant was similar to the Δ*hyd*1 knockout, although its surface actually appears less smooth than the Δ*hyd*1 mutant with some surface structures noticeable.

Aerial conidia of filamentous fungi, including *B. bassiana* are coated by hydrophobins. Although few studies have examined this process, it is thought that the rodlet layer is formed by spontaneous self-assembly of the hydrophobins on the cell surface. Previous results indicated that the primary component of the sporecoat to be Hyd2 (Holder & Keyhani, 2005), but analysis of the Δ*hyd*1 and Δ*hyd*2 mutants suggest a role for both proteins in the spore coat rodlet layer. In order to provide more information regarding the relationship between Hyd1 and Hyd2 on the surface of aerial conidia,

attempts were made to complement the Δ*hyd*2 conidial phenotype with recombinant Hyd2 protein solution added to *B. bassiana* fungal spores in solution. This "trans-complementation" of the *hyd*2 knockout with a nHyd2 protein solution resulted in the appearance of patches of rodlet filaments, ie a partial or complete restoration of the phenotype (Fig. 2-15). The trans-complimented conidial spore coat contained patches of ordered arrays of striated bundles (L = 93.5 ± 31.6 nm, W = 24.7 ± 8.0 nm, 4.2 ± 1.5 striations/bundle; N = 5 cells, 20 bundles each) similar to that seen on the wild type conidia. Experiments in which nHyd2 was added to Δ*hyd*1 conidia had no apparent effect on the morphology of the spore coat. Trans-complementation appeared to depend upon the age of the conidia harvested from PDA plates. Conidia that were >28 day old appeared to be unable to be trans-complemented. In order to better define the conditions allowing for the trans-complementation to occur, a time course using Δ*hyd2* conidia from the same plate at 10, 14, 21 and 30 days were incubated with purified nHyd2. These data revealed that optimal trans-complementation occurred between 10 and 14 days, whereas days 21 and 30 were unable to be trans-complemented (Fig 2-16). Δ*hyd2* conidia were then trans-complemented at increasing pH's from 4.0-10.0 to determine possible effect of ionic strength upon trans-complementation (Table 2-5). Δ*hyd2* conidia harvested at day 14 showed an inability to be trans-complemented at pH's above 8.0; whereas at day 30 the Δ*hyd2* conidia could only be trans-complemented at a pH of 4.0. To assess whether the observed inability of trans-complementation was due to the effect of pH on nHyd2 self assembly, the nHyd2 was monitored for self-assembly by ThT assay over a pH range of 4.0-10. Results indicate that the pH did not inhibit self-assembly (data not shown).

In order to examine whether the trans-complementation depended upon the living state of the conidia, Δhyd2 conidia were heat killed, glutaraldehyde fixed, or UV exposed prior to being incubated with the purified nHyd2 protein solution. The conidia were then imaged by AFM using contact mode as previously described (Fig. 2-17). No complementation was observed after any of the treatments described above.

Discussion

Surface Characteristics

Fungal cells display a wide range of surface physicochemical properties that allow them to interact and adhere to substrata. Cell surface hydrophobicity is associated with increased virulence of *Candida* strains and the hydrophobic rodlet layer of *Aspergillus* conidia appears to confer protection against specific host immune reactions (Hazen, 2004; Paris *et al.*, 2003; Singleton *et al.*, 2005). The determination of surface biophysical features of *Aspergillus* spores has revealed a role for the rodlet layers and their hydrophobin constituents in contributing to hydrophobicity, adhesion and resistance to killing by alveolar macrophages of the fungal cells (Dynesen & Nielsen, 2003; Girardin *et al.*, 1999b; Paris *et al.*, 2003; Stringer & Timberlake, 1995; Thau *et al.*, 1994). The surface hydrophobin RodA was found to prevent host immune recognition of airborne fungal spores in *A. fumigatus* (Aimanianda *et al.*, 2009). Disruption mutants of RodA did not induce dentritic cells or alveolar macrophages, and could not activate helper T-cells *in-vivo* (Aimanianda *et al.*, 2009). However, care should be taken in the interpretation of this immunological inertness, as healthy individuals are immune to infection by *A. fumigatus*.

Entomopathogenic fungal spore surfaces range from hydrophobic, exemplified by fungi such as *Nomuraea rileyi*, *Metarhizium anisopliae* and *Paecilomyces*

fumosoroseus, all of which possess defined outer rodlet layers, to hydrophilic, as seen in *Hirsutella thompsonii* and *Verticillium lecanii*, often characterized by the lack of a rodlet layer but containing an outer mucilaginous coat produced during spore maturation (Boucias & Pendland, 1991). AFM has been used to visualize the surface features of live fungal cells (Dufrene, 2000; Holder & Keyhani, 2005; Zhao *et al.*, 2005). Other methods such as electron microscopy are limited by the requirement of fixation and sample processing which precluded visualization of living cells. AFM, however, allows for a real time imaging of the surface characteristics which is essentially noninvasive and results in scanning and imaging at nanoscale resolutions. High-resolution AFM micrographs of *Aspergillus nidulans* have revealed alterations to the spore rodlet layer during swelling in aqueous solutions, and ultrastructural features of newly deposited walls at hyphal tips as well as in mature walls (Ma *et al.*, 2005; Ma *et al.*, 2006). In the present study, AFM was used to examine surface morphological differences among the different *B. bassiana* cell types that were air-dried 3-4 hours before imaging. Initial experiments using an aqueous AFM cell chamber resulted in images that were not as clear as the ones presented, although future experiments imaging the cell types in an aqueous environment are warranted. *B. bassiana* aerial conidia contained fascicle bundles that were not present on either submerged conidia or blastospores. Surfaces of blastospores were smooth, whereas those of submerged conidia were more granulated in appearance. These results are consistent with the lack of SDS-insoluble, trifluoroacetic acid (TFA)-soluble proteins in the latter two cell types and their presence in aerial conidia (Holder & Keyhani, 2005). Furthermore, no fascicles were visible on

germ tubes emanating from aerial conidia, suggesting little rodlet layer fluidity between spores and the growing germ tube.

Hydrophobicity and zeta potential measurements have been used to predict the binding preferences of the fungal cells. Analysis of the cell surface properties of *Cryptosporidium* oocytes has revealed a preference for adhesion to glass rather than hydrophobic plastic materials, although cell surface hydrophobicity increases with increasing ionic strength of the medium (Drozd & Schwartzbrod, 1996). In contrast, conidia of the mycoparasite *Coniothyrium minitans* are hydrophobic, although in this case, conidial hydrophobicity decreases with culture age for some isolates (Smith *et al.*, 1998). Similarly, a comparison of two cell types of *Trichoderma harzianum*, a potential biological control agent of phytopathogenic fungi, reveals that aerial conidia display higher UV resistance and longer viability, and are more hydrophobic than submerged conidia, which are hydrophilic (Munoz *et al.*, 1995). Contact angle measurements, microbial adhesion to solvents and zeta potential determinations of blastospores of the entomopathogenic fungus *P. fumosoroseus* indicate that these cells have a hydrophilic, basic monopolar surface, and are negatively charged under neutral conditions (Dunlap *et al.*, 2005).

Hydrophobicity was examined using two different methods: partitioning of cells in organic vs. aqueous solvents (MATH), and hydrophobic index chromatography (HIC). The first two methods gave almost identical HI values for the cells tested. Overall, aerial conidia are hydrophobic and represent the more resistant spore type, but are slower growing than blastospores and submerged conidia. Hydrophobic interactions predominate in the case of aerial conidia and are likely to be the most important force in

the host–pathogen interaction. Although they are hydrophobic, the net negative surface charge of aerial conidia at neutral pH may help account for their ability to bind hydrophilic surfaces weakly (Holder & Keyhani, 2005). A negative shift in the surface electrostatic charge distribution was noted for aerial conidia as they aged (Holder *et al.*, 2007; Smith *et al.*, 1998). This could be due to the production of increasing amounts of surface anionic species over time, or the unmasking of negative charges as the spores dry. Similar experiments could not be performed with blastospores and submerged conidia, as these cells are not stable and will either grow (i.e. germinate or microcycle conidiate) if sufficient nutrients are present or lose viability over a similar time-course.

The surface charge distribution of these cells is consistent with their ability to bind weakly polar and hydrophilic substrata (Holder & Keyhani, 2005). The submerged conidia display intermediate surface properties in terms of hydrophobicity and electrostatic charge. These cells can grow under nutrient-limiting conditions and are likely to exist on insect surfaces and during host–parasite competition for nutrients. The intermediate surface hydrophobicity values reported in this study again may help account for the adhesive nature of these cells, which were able to bind to hydrophobic, weakly polar and hydrophilic surfaces (Holder & Keyhani, 2005; Holder *et al.*, 2007).

The production of different infectious propagules and the wide range of their surface properties imply a diversification of adaptations evolved by fungal pathogens in mediating attachment and adhesion to target insect surfaces. The ability to produce more than one spore type with different surface properties can be expected to increase the possibility of binding diverse ranges of substrata. Biological control applications of entomopathogenic fungi, including *B. bassiana*, often employ aerial conidia as the

infective agent; however, the use of other single-cell propagules, such as blastospores and submerged conidia, has also been attempted.

cDNA Cloning and Expression Analysis of Hydrophobins

Hydrophobins are unique fungal proteins that function in a diverse array of physiological processes. Since hydrophobins are often highly expressed, the isolation of genes encoding these proteins has largely been accomplished from genomic [e.g. expressed sequence tag (EST)] analyses or, in some cases, by purification of the protein product and stepwise cloning of genes using nucleotide primers based upon the available amino acid sequence data (Wessels, 1997; Wessels, 1999; Wosten & de Vocht, 2000).

In *B. bassiana*, the *hyd1* transcript was indeed highly expressed and found in our own EST libraries (Cho *et al.*, 2006b). The *hyd2* gene, however, was isolated by phage display (Cho *et al.*, 2007a). The translated amino acid sequences of *hyd1* and *hyd2* did not match the 16 aa N-terminal sequence of a putative hydrophobin isolated from *B. bassiana* reported elsewhere, although the N terminus of mature Hyd*2* did show some homology (~50 %) to the reported protein (Bidochka *et al.*, 1995b). Similarly, although not an exact match, the N terminus of mature Hyd1 showed homology (~40 %) to the N terminus (as determined by amino acid sequencing) of what was termed an inner cell wall protein (cwp1) of *B. bassiana* (Bidochka *et al.*, 1995a). Expression analysis indicated that *hyd1* was highly expressed under almost all growth conditions examined. These results are consistent with previous EST analyses of *B. bassiana* grown under different developmental conditions (Cho *et al.*, 2006a; Cho *et al.*, 2006b). Notably, *hyd1* transcript was abundant in all the single-cell spore types (aerial conidia, *in vitro* blastospores and submerged conidia), whereas *hyd2* transcripts were essentially

absent from these cells. Thus, even though the spore coat of aerial conidia is composed of the Hyd2 protein, no *hyd2* transcript was detected in these cells. This is perhaps not too surprising, since it is the aerial conidia that need to make *hyd2* and its protein product, and therefore one would not expect to see *hyd2* transcript in the final product of this process, namely the aerial conidia themselves. Transcripts corresponding to *hyd2* were also detected in cells grown in 1 % chitin or 1 % insect cuticle, but were not detected in cultures containing chitin + cuticle, each at 1 % concentration. This result was observed in two separate biological samples in which actin was used as a control. Although it is unclear why *hyd2* transcript was not detected under the latter growth conditions, it could be due to the relative nutrient levels in the cultures. Cells growing under 1 % chitin or 1 % cuticle conditions may be nutrient-limited, which would result in sporulation (i.e. conidiogenesis and hence *hyd2* transcript production), whereas cultures containing both nutrient sources may grow in a vegetative state for a longer period of time. Further experiments are necessary to determine how these hydrophobins genes are developmentally regulated.

Hydrophobin Production and Purification

The unique biophysical properties of hydrophobins i.e. their ability to form an amphipathic film on an array of surfaces, make them an interesting target for directed biotechnical applications. In order to fully explore these applications large quantities of the protein are required. However, one of the challenges in working with class I hydrophobins has been their production and purification. A number of class II hydrophobins such as HFBI and HFBII of *Trichoderma reesei* and SC3 of *Schizophyllum commune* have been expressed at levels relatively high (> 200 mg/L)(Askolin *et al.*, 2001). It is thought that there are foldases and chaperones that are

necessary for correct folding of class I hydrophobins (Hektor & Scholtmeijer, 2005), therefore current production of hydrophobins relies on either extracting the rodlet layer directly from the fungi or by expression of the protein in yeast. This method is time consuming and may be contaminated with host hydrophobins. However, the class I hydrophobin from *Neurospora crassa*, EAS, has been expressed and purified from *E.coli* (Kwan *et al.*, 2006). Here we utilize the *E.coli* intein purification system to allow for fast and efficient production of the *B. bassiana* Hyd2 protein. Similar to many eukaryotic proteins expressed in *E.coli*, and undoubtedly due to their unique structural characteristics, our results indicate the nHyd2 forms insoluble inclusion bodies in the *E. coli* host. Purification from the inclusion bodies involved a stepwise refolding strategy and use of an intein fusion partner for purification. The use of an intein based affinity purification system was originally developed as a way of expressing proteins in which the mature protein product would not contain any extraneous tags that could limit protein activity (Xu *et al.*, 2000). In order to make use of the autocatalytic activity of the intein, we resorted to a specific amino acid replacement on the N-terminal of the Hyd2 protein. The first three amino acids ALA, PRO, and HIS, which due to structural constraints reduce intein autocatalytic activity, were replaced with GLY, GLY and ALA respectively. The amino acid replacement allowed for optimal autocatalytic conditions for the removal of the intein fusion moiety. Our results indicate that (i) addition of a pH induced self cleavable intein fusion partner and (ii) a step wise refolding of the class I hydrophobin inclusion bodies results in the yield of a reasonable amount of protein (5-10 mg/L). The expression and purification strategy described above has several advantages over traditional expression strategies. There is no need of a secondary

enzymatic cleavage reaction to remove the fusion partner, self cleavage of the intein moiety results in no additional amino acids, and coexpression with a large fusion moiety can prevent any proteolytic degradation (Hong *et al.*, 2001). Also, the successful removal of the intein in which there is a cysteine on the N-terminal of the protein would generate the correct precursor for intein mediated protein ligation. This would allow the ligation of the target protein to a thioester group of additional peptide fusion partners; or the target protein can be hydrolyzed to reveal the free amino terminus, which allows for versatile usage of the protein with minimal preparation.

nHyd2 Self-assembly

A primary biophysical property of hydrophobins is their ability to self-assemble into a monolayer at hydrophobic/hydrophilic interfaces (Hektor & Scholtmeijer, 2005; Kwan *et al.*, 2006; Linder *et al.*, 2005; Szilvay *et al.*, 2007). Reself-assembly of hydrophobins obtained directly from the fungal surface has been demonstrated in many fungi, including the *Neurospora crassa* EAS, *Grifola frondosa* HGFI, *Schizophyllum commune* SC3, and *Trichoderma reesei* HFBI (Beckerman & Ebbole, 1996a; Bellpedersen *et al.*, 1992; Hakanpaa *et al.*, 2006; Hektor & Scholtmeijer, 2005; Lugones *et al.*, 1996b; Paris *et al.*, 2003; Szilvay *et al.*, 2007; van Wetter *et al.*, 2000). Any system used to produce and purify these proteins must maintain this key ability. Several lines of evidence were used to determine whether the recombinant nHyd2 protein retained its activity of self-assembly. These included ThT assay, LB isotherms, AFM, SEM, surface tension, and Water Contact Angle (WCA). The results of each of these experiments lead to the conclusion that the nHyd2 retained its native activity.

The presumptive self-assembly assay of the recombinant nHyd2 protein was determined via thioflavin-T (ThT) fluorescence. Thioflavin-T is an aromatic dye that

binds specifically to stacked β-sheet structures. It is commonly used in the diagnosis of amyloid fibrosis in which the stacked β-sheet structures of the amyloid plaques can be detected via binding of the ThT. Hydrophobins have been compared to amyloid fibrils and the ThT assay as been applied for their characterization (Sunde *et al.*, 2008). Hydrophobins exist in three structural states: a monomeric state which is soluble, an α-helical state, and a β-sheet state when the protein has self assembled at hydrophobic/hydrophilic interfaces such as the water/air interface (de Vocht *et al.*, 2000). The Thioflavin-T fluorescence assay allows for a rapid screening of the ability of a hydrophobin to self-assemble into 2-dimensional arrays. Thioflavin-T selectively binds to proteins with stacked β-sheet amino acids, when the hydrophobin self-assembles, it changes confirmation to a primarily β sheet state which then can bind to the Thioflavin-T. Using this assay we were able to confirm functional self-assembly of the purified hydrophobin. The ThT assay indicated that the Hyd2 protein self-assembles over time when agitated was revealed by the increased fluorescence. Vigorous agitation maximizes water-air interaction of the hydrophobin leading to assembly, a fact often exploited in their purification (De Stefano *et al.*, 2007). Also, our results indicated that there might be a concentration threshold to maximize the self-assembly process. A decrease in concentration from 150ug/ml to 75ug/ml showed significant decrease in the RFU's (650 to 250) over a 7 minute period. This could be due to a minimum concentration that is required to initiate the self-assembly.

Atomic force microscopy has widely been used to image a broad array of surfaces due to its noninvasive sample preparation and imaging technique. It is commonly used to visualize the surface of fungi and hydrophobin monolayer preparations (Dague *et al.*,

2008; De Stefano *et al.*, 2007; de Vocht *et al.*, 2000; Hakanpaa *et al.*, 2004). In this work we used AFM determine self-assembly and the homogeneity of the protein as it assembles on a glass substrate. Our micrographs confirmed the presence of a layer of hydrophobins a few nanometers thick. The hydrophobin monolayer seemed to be compact with few holes or irregularities in the areas covered. This is consistent with previous results showing films of homogeneous preparations of hydrophobin monolayers on different surfaces (de Vocht *et al.*, 2000; Hakanpaa *et al.*, 2004). AFM and TEM were used to visually confirm the presence of a hydrophobin monolayer. This indicates that the hydrophobin can be used as a surface coating without discontinuities in the monolayer. This rigid monolayer is similar to other types of self assembling molecules such as S-Layer proteins found on the surface of a large number of bacteria and Archaea (Martin-Molina *et al.*, 2006). The amyloid-like filaments found after TEM analysis are similar to those found in AFM and SEM micrographs of HGFI *and* EAS hydrophobins (Mackay *et al.*, 2001; Yu *et al.*, 2008).

Water contact angle was used to study the surface modification ability of the recombinant *B. bassiana* nHyd2 protein. Hydrophobins have the characteristic of being able to alter the “wettability” of surfaces, i.e. they can alter a hydrophobic substrate to a more hydrophilic substrate. This has been demonstrated for several class I hydrophobins including the *Grifola frondosa* HGFI and *Schizophyllum commune* SC3 (Askolin *et al.*, 2006; Hou *et al.*, 2009; Yu *et al.*, 2008). For example, a siliconized glass surface on which HGFI had been deposited resulted in the alteration of the water contact angle from 86.6^{o} to 51.9^{o} indicating an increase in surface hydrophilicity. They also reported a sharp decrease in the surface tension with increasing concentrations of

HFGI (specifically between 1 and 2 uM) (Yu *et al.*, 2008). Advancing and receding contact angles on nHyd2 protein solutions were measured, and both measurements show a decrease in contact angle of roughly 30^{o} on glass slides. This indicates that the hydrophobin solution is altering the wettability characteristics of the glass slide making it more hydrophilic.

Trans-Complementation of Δ*hyd2*

During fungal development, hydrophobins have been shown to have an array of different physiological roles extending from mediating cell-substrate attachment, lowering of liquid surface tension to allow growth, and as an element of the fungal spore coat structure (Bidochka *et al.*, 1995b; de Vocht *et al.*, 1998; Ebbole, 1997b; Fuchs *et al.*, 2004; Hektor & Scholtmeijer, 2005; Holder *et al.*, 2007; Houmadi *et al.*, 2008; Kazmierczak *et al.*, 2005a; Linder *et al.*, 2005; Lugones *et al.*, 1996b; Paris *et al.*, 2003). Gene knockouts of the class I hydrophobin MPGI from *Magnaporthe grisea* resulted in reduced pathogenicity due to reduced attachment, fewer appressorium, and increased wettability (Talbot *et al.*, 1996). Self-assembly studies have shown that class I hydrophobins readily form rodlets *in-vitro*; however these rodlets are primarily seen after compression with a Langmuir Blodget system (Houmadi *et al.*, 2008). Little is known, however, regarding the mechanisms by which the rodlets are generated on the surface of the conidia. In the lab, knockouts of *hyd1* and *hyd2* were constructed to determine their function and help characterize the self-assembly process on the spore surface (S. Zhang and N.O. Keyhani, unpublished results). The surface of Δ*hyd1* aerial conidia showed a relatively smooth surface while the Δ*hyd2* mutant had cell surfaces which appeared disorganized. It was originally hypothesized that the double mutant Δ*hyd1*Δ*hyd*2 would have a surface structure similar to the of the Δ*hyd1* mutant,

however, it had a surface structure that was distinct from both the Δ*hyd*1 and Δ*hyd*2 mutants, implying that loss of both proteins may result in the exposure or uncovering of some underlying cell structures. My current hypothesis is that both Hyd1 and Hyd2 act cooperatively to produce the rodlet layer with Hyd2 acting as an organizational factor. We conclude that although a rough sporecoat can be formed without Hyd2, the conidia cannot complete the formation of the striated rodlet layer. Previous studies of rodlet proteins in *A. fumigatus* showed that deletion of the rodB hydrophobin had no effect upon rodlet assembly, but ΔrodA results in a spore surface completely void of rodlets. In addition, ΔrodAΔrodB mutants also showed a removal of the rodlet layer but was not similar to that of the ΔrodA mutant (Dague *et al.*, 2008), similar to our results in *B. bassiana.*

Using purified recombinant nHyd2, a trans-complementation of Δ*hyd1* and Δ*hyd2* was assayed on 14-30 day old conidia. These experiments showed that a solution of purified Hyd2 was able to complement the Δ*hyd*2 morphological phenotype. The restoration seemed to occur in patches on the surface of the spore as indicated by formation of the striated bundles of rodlets that is characteristic of the wild-type sporecoat. As expected, the trans-complementation of the wild-type and Δ*hyd*1 had no apparent affect upon the surface morphology of the spore. Furthermore, Δ*hyd*2 conidia were unable to be trans-complemented when the spores were harvested from agar plates that were over 21 days old. Previous studies on *Coniothyrium minitans* conidial age versus electrostatic charge reveals that as conidia age they become more negatively charged to a maxima after ~34 days (Smith *et al.*, 1998). These results would correlate well with the expression pattern of *hyd2* in which the gene seems to

only be expressed at the beginning of conidiogenesis where presumably the cell surface would have the least negative charge. To determine the effect of ionic strength associated with nHyd2 assembly on the conidial surface, the Δ*hyd*2 (harvested at 14 and 30 day) were trans-complemented at increasing pH from 4.0 to 10.0. Results indicate that there is an optimal pH (pH 5-7) required for trans-complementation to occur. Previous results have shown that as the pH of a conidial suspension increases, there is a decrease in surface charge (Holder *et al.*, 2007). It is possible, therefore, that the cell surface charge may influence the self-assembly of hydrophobins on the cell surface. To our knowledge this is the first report of a recombinant hydrophobin protein being used to complement or regenerate the rodlet layer. Trans-complementation of heat killed, glutaraldehyde fixed, and UV exposed Δhyd2 conidia indicated that Hyd2 cannot complement nonliving cells. These manipulations may have destroyed a critical secondary component needed for assembly of Hyd2 or the process may require energy or some co-factor. Further experiments are needed to define the parameters that determine hydrophobin assembly in living spores.

In conclusion, the development of an efficient expression and purification system for functionally active nHyd2 has been developed. Production of functionally active recombinant Hyd2 allows for an in-depth study of the self-assembly process on the surface conidia. The current hypothesis is that Hyd1 and Hyd2 act cooperatively to produce the rodlet layer of aerial conidia with Hyd2 acting as an organizational factor. It has also been shown that trans-complementation is an effective method for looking at the self-assembly of the nHyd2 (and Hyd1 in the future) into the rodlet layer. Finally, results show that the self-assembly of nHyd2 on the surface of Δ*hyd*2 conidia is surface

charge dependent. Future work will include the expression and purification of recombinant Hyd1 as well as trans-complementation studies using both nHyd1 and nHyd2.

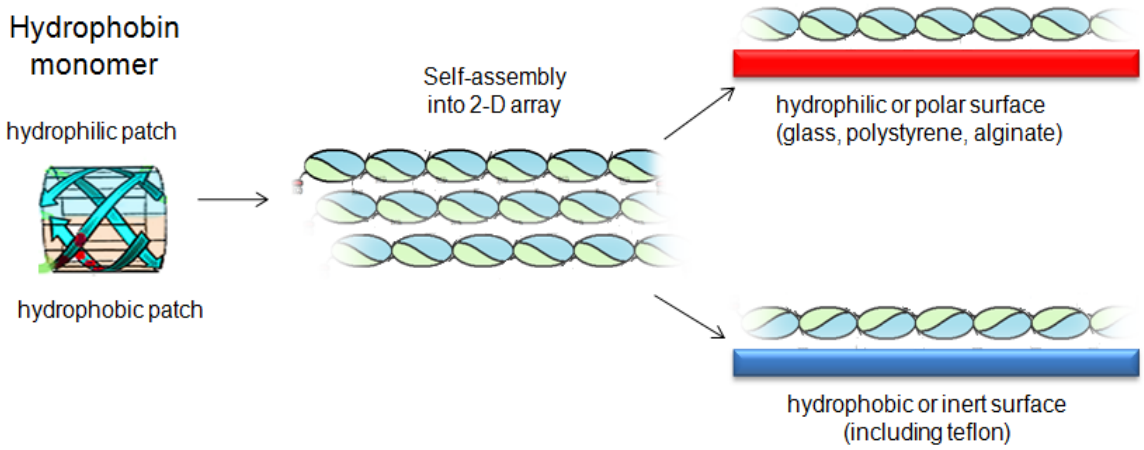


Figure 2-1. Possible model for hydrophobin rodlet formation. Schematic representation of hydrophobin monomers stacking at an hydrophobic or hydrophilic interface.

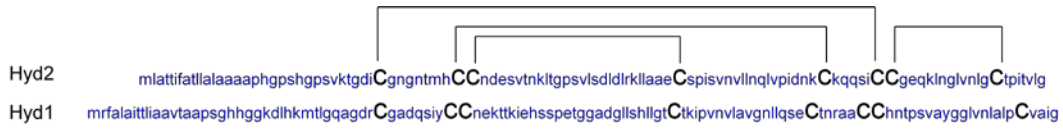


Figure 2-2. Sequence of Hyd2 and Hyd1, indicating conserved disulfide bonding pattern. Disulfide bonds form between Cys^{1}-Cys^{6}, Cys^{2}-Cys^{5}, Cys^{3}-Cys^{4}, and Cys^{7}-Cys^{8}.

Table 2-1. Primer sequences and product sizes for semi-quantitiative RT-PCR F, forward; R, reverse.

Gene Product	Primer Sequence 5'-3'
Hyd1	F: caccatggtggaaaggatctgcac
	R: ccgagaaggtgggaaagaagacca
Hyd2	F: tgtcaagactggcgacatttgcg
	R: tcgatggggacaagctggttga
β-Tubulin	F: tccttcgtacggtgacctga
	R: cgagcttgcgaagatcagag
Actin	F: ttggtgcgaaacttcagcgtctagtc
	R: tccagcaaatgtggatctccaagcag

Table 2-2. List of primers used in this study

Primer Name	Sequence
Hyd2	F: ggtggttgctcttccaacggcggcgctggccgcggacgcagccacggccgcagt
	R: ggtggtctgcagttatccgaggacggtgat
BK11	F1: gtgaaagtgaaagctccccacggacccagccacggc
	F2: ggtggttgctcttccaacttcaaggtgaaattcaaggtgaaagtgaaa
	R: ggtggtctgcagttatccgaggacggtgat
BK14	F: ggtggttgctcttccaacggcggcgctggccgcggacgcagccacggccgcagt
	R1: caccttgaatttcaccttgaatccgaggacgttgatggg
	R2: ggtggtctgcagttacttcactttcaccttgaatttcaccttgaa
BK15	F: gtgaaagtgaaagctccccacggacccagccacggc
	R: ggtggtctgcagttacttcactttcaccttgaatttcaccttgaa
CM4	F1: cgctggaagatctttaaaaaaattgaaaaagtgggtcaaaacatcagagacggcatcg
	F2: ggtggtcatatgcgctggaagatctttaaaaaaatt
	R1: gatggtggcagcttgacctacaacagctactgctggaccagcttttacgatgccgtctc
	R2: ggtggttgctcttccgcagatggtggcagcttgacctacaac
cysHyd2	F: ggtggttgctcttccaactgcggcggcgctggccgcggacgcagccacggccgcagt
	R: ggtggtctgcagttatccgaggacggtgat

Forward and revese primers for the production of hyd2 construct, BK11 which has FKVKFKVKVK on Nterminus, BK14 which has FKVKFKVKVK on C-terminus, BK15 which has FKVKFKVKVK on both termini, CM4 antimicrobial peptide, and Hyd2 with an Nterminal cysteine for IPL reaction.

Table 2-3. Buffer concentrations for refolding Hyd2 protein from inclusion bodies

I	8M Urea, 10mM DTT, 20mM Tris, 300mM NaCl
II	6M Urea, 1mM DTT, 20mM Tris, 300mM NaCl
III	4M Urea, 1mM DTT, 20mM Tris, 300mM NaCl
IV	2M Urea, 1mM Reduced Glutathione, 20mM Tris, 300mM NaCl
V	1mM Reduced Glutathione, 20mM Tris, 300mM NaCl

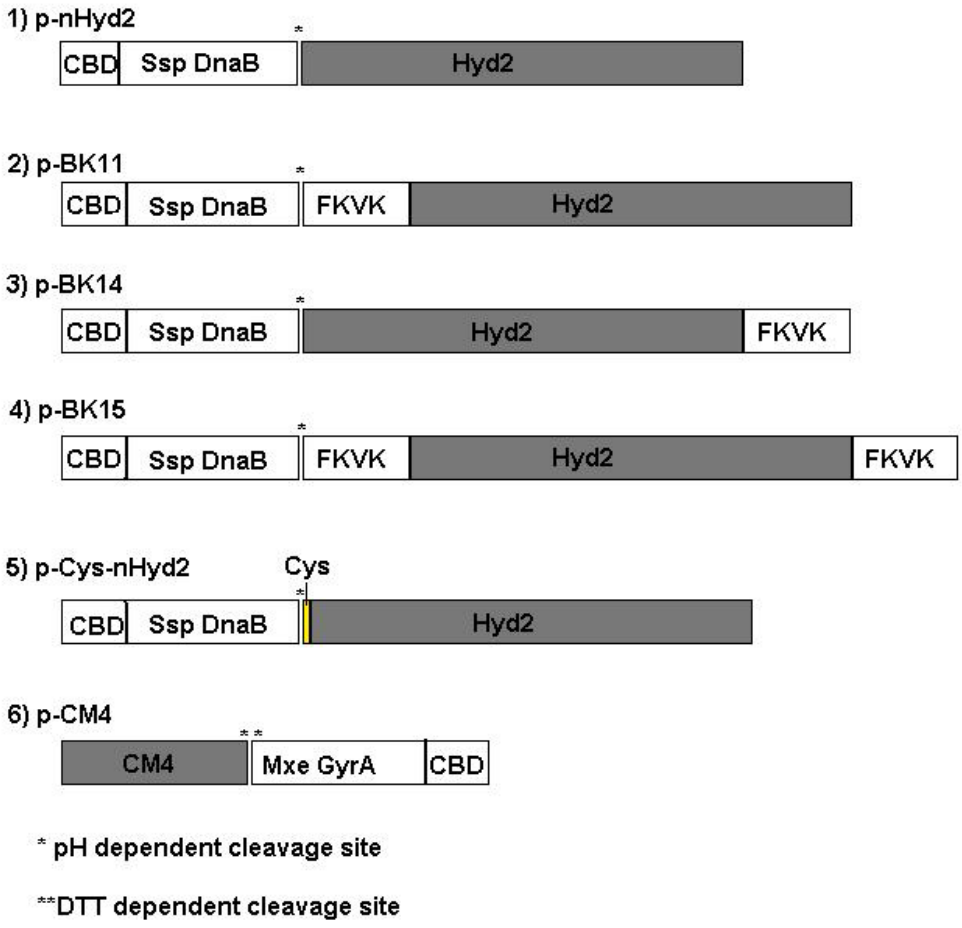


Figure 2-3. Vector constructs of nHyd2 and nHyd2 derivatives (see appendix).

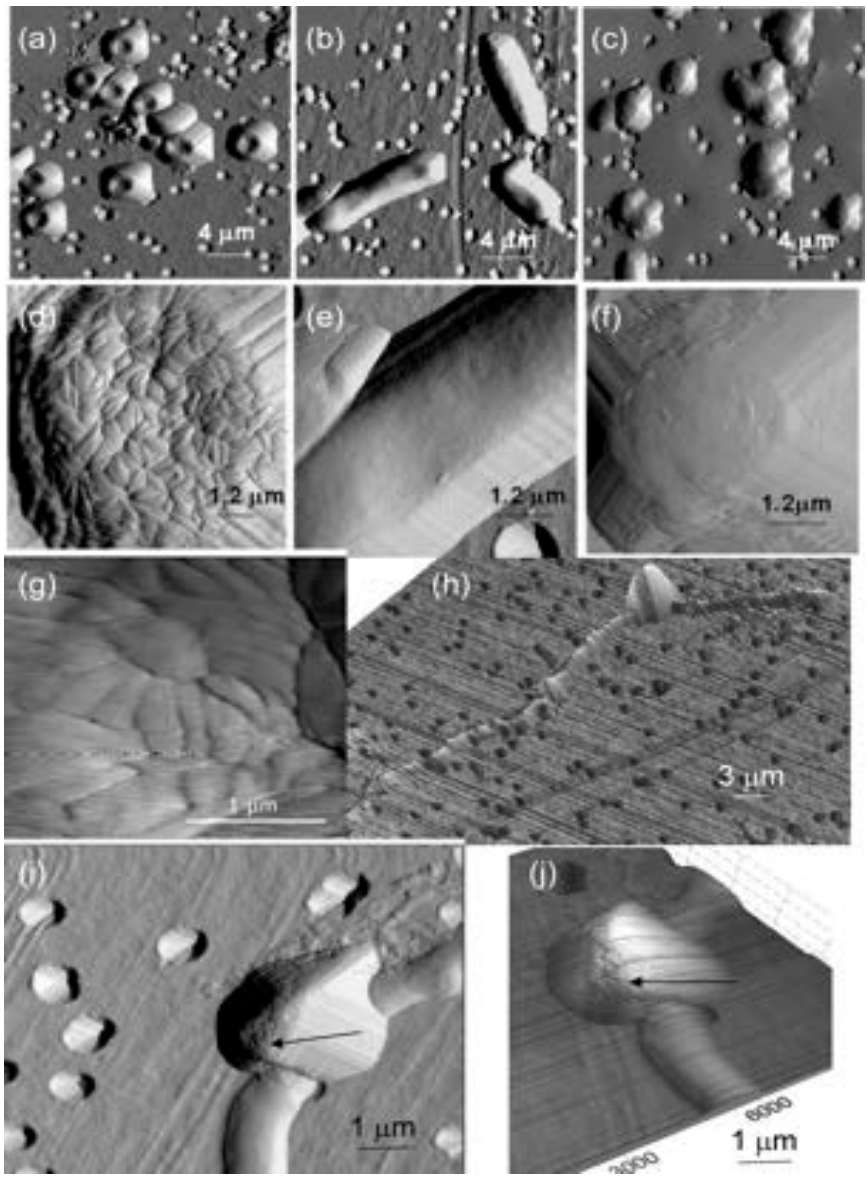


Figure 2-4. Atomic force micrographs of *B. bassiana* spore types and germinating conidia. (a, d, g) Aerial conidia: note surface fascicles presumably composed of hydrophobin rodlets [rodlet filaments faintly visible in (g)]; (b, e) *in vitro* blastospores; (c, f) submerged conidia; (h) bipolar germination of aerial conidia. (i, j) Higher resolution of germinating aerial conidia: note the fascicles still present on the germinated conidia (arrows).

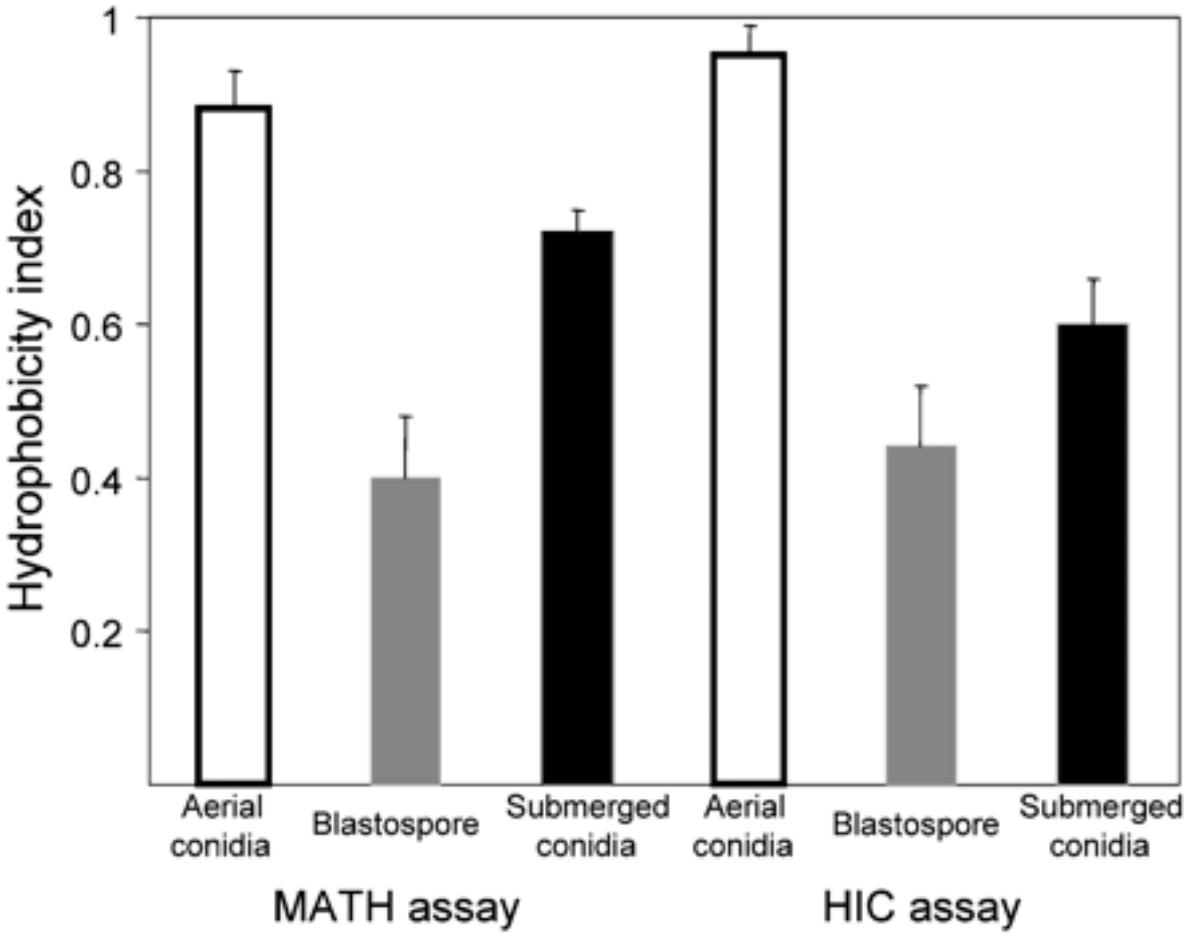


Figure 2-5. Cell surface hydrophobicity of the three *B. bassiana* spore types assessed by MATH assay and HIC.

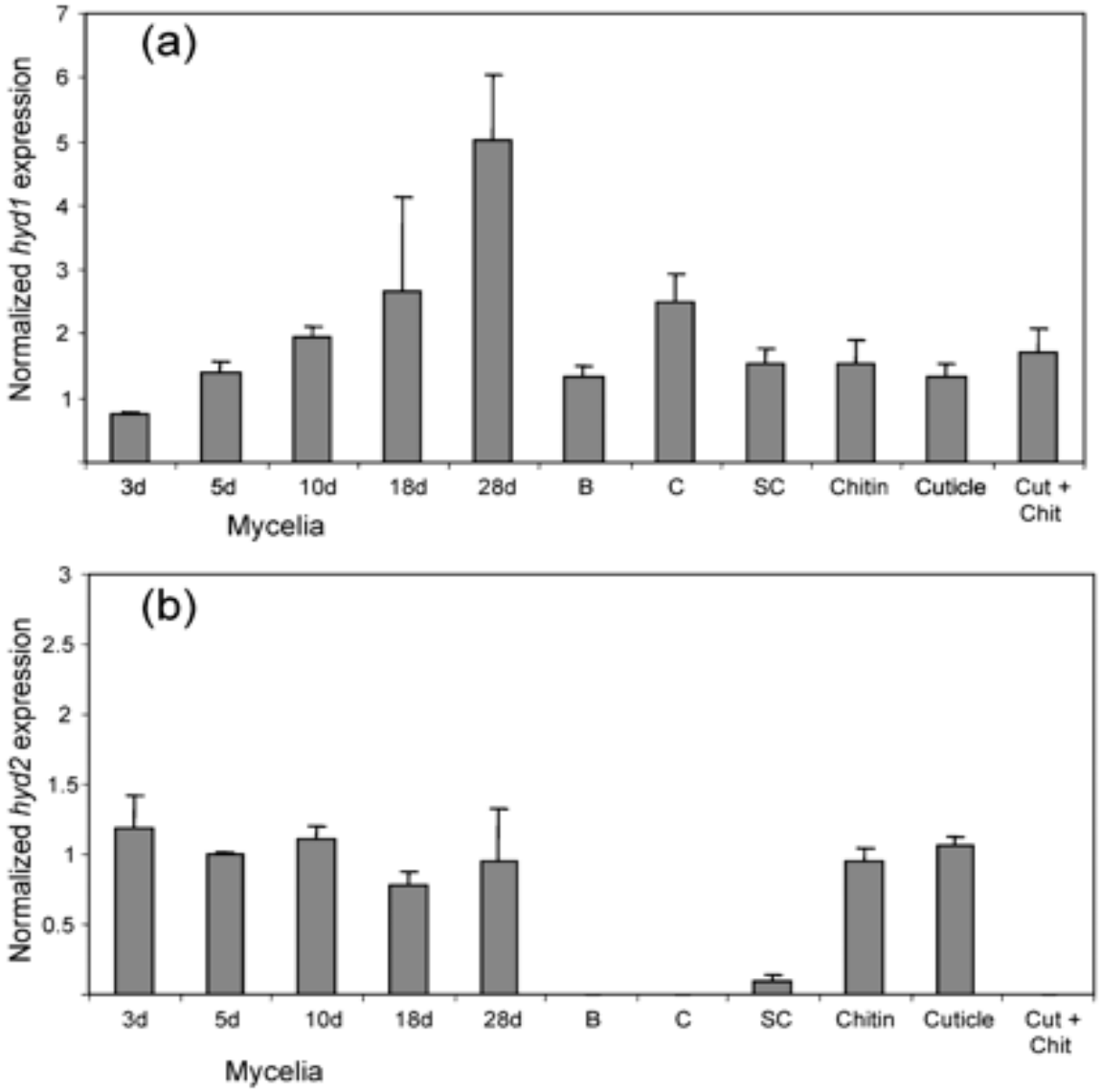
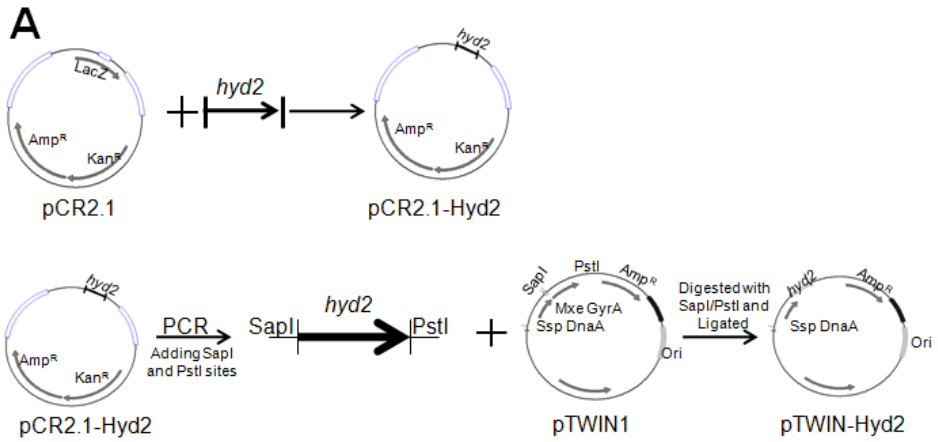


Figure 2-6. Expression analysis of *hyd1* and *hyd2*. Semi-quantitative RT-PCR was used to determine the expression of the *hyd1* (a) and *hyd2* (b) genes in various *B. bassiana* cell types and during specific developmental conditions, as described in Methods. Only data within the linear portion of PCR amplification were considered valid, and the data shown are derived from 25 cycles of PCR amplification. Actin expression was used as an internal control for normalization of the data (similar results were obtained using *β*-tubulin). Expression of the hydrophobin and actin genes was examined during mycelial growth on PDA plates from which conidia were removed (3, 5, 10, 18 and 28 days), and from liquid cultures of blastospores (B), aerial conidia (C), submerged conidia (SC), and after 3 days of growth in the presence of chitin (1 %), insect cuticle (1 %), or chitin+insect cuticle (Cut+Chit; 1 % each).



B

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301  taatgaacag acgatgaagc tagaatcagc taaagttagt cgtgtatttt gtactggcaa aaagctagtt tatattctaa aaactcgact aggtagaact atcaaggcaa cagcaaatca tagatttta actattgatg gttggaaaag
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751  tgacgagtct gtcaccaaca aactcactgg ccccagcgtt ctcagcgacc tggatctccg caagcttctc gccgccgagt gcagccccat ctccgtcaac gttcttctca accagcttgt ccccatcgac aacaagtgca agcagcagag
901  catttgctgt ggcgagcaga agcaaaacgg tctcgtcaac cttggctgca ctcccatcac cgtcctcgga taactgcagg aaggggatcc ggctgctaac aaagccgaa aggaagc
```

Figure 2-7. A) Vector construction of the hyd2 gene inserted into pTWIN1 vector. The hyd2 gene is first subcloned into the pCR2.1 vector and then cloned into the pTWIN1 vector. Ssp DnaB is directly upstream allowing its N-terminal fusion during expression. B) Sequence confirmation of hyd2 inserted into pTWIN1 vector. Red is Ssp DnaB intein and Blue is hyd2 gene.

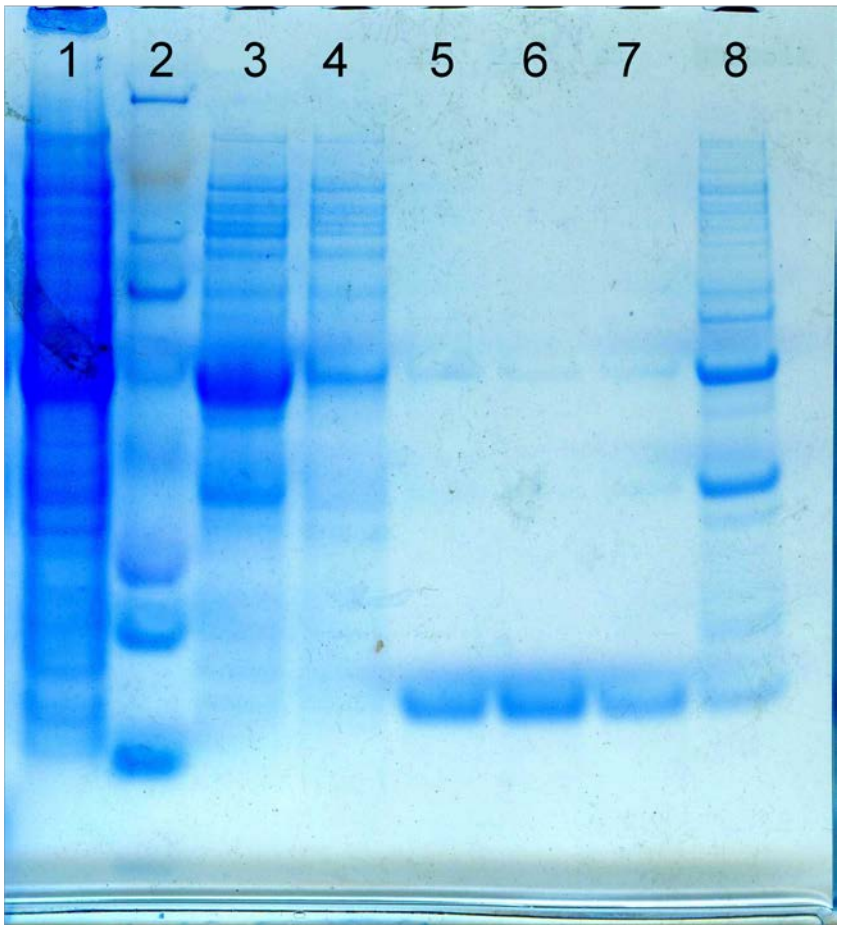

Figure 2-8. LDS PAGE analysis of purified nHyd2 protein (lanes 5-7) using 12% bis-tris gel after refolding and cleavage from Ssb DNaA N-terminal intein fusion partner. Induced crude extract, Lane 1; Molecular Weight marker, Lane 2; Crude extract after refolding protocol, lane 3; Flowthrough, Lane 4; Chitin beads boiled in SDS, Lane 8.

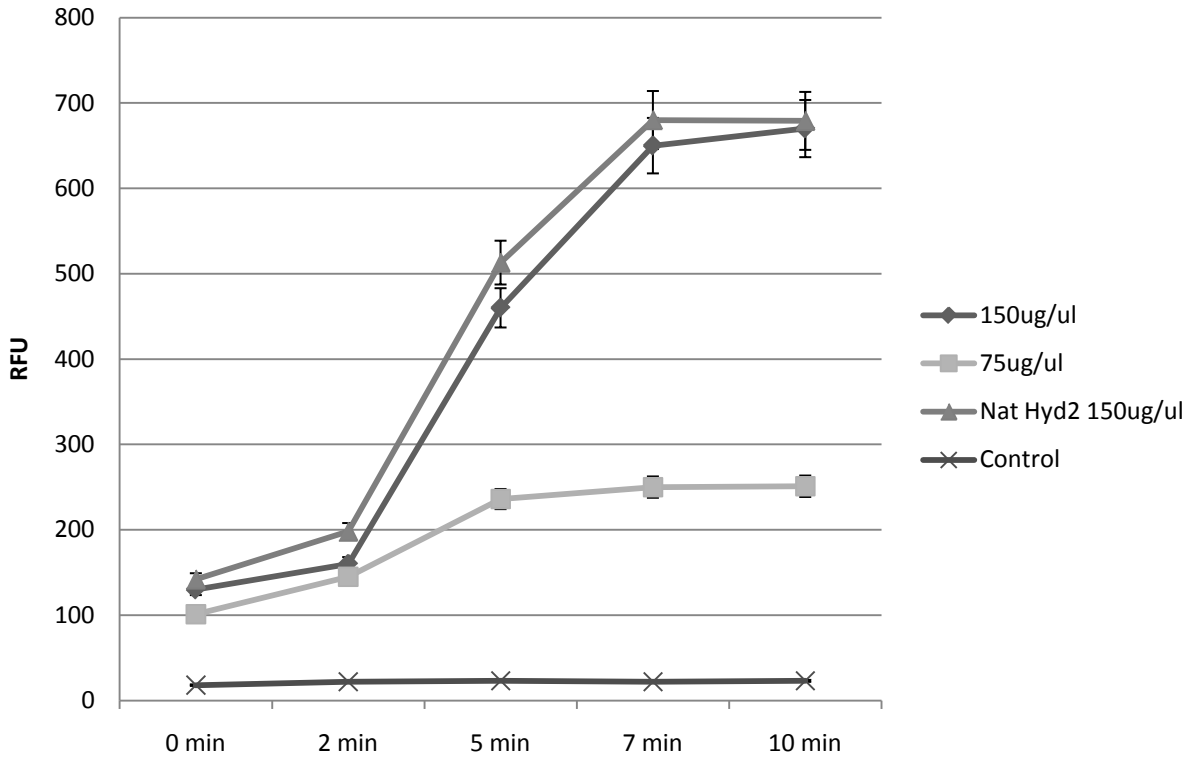

Figure 2-9. nHyd2 rodlet formation timecourse as monotered by ThT binding. 150 and 75 ug/ml nHyd2 and 150ug/ul Native Hyd2 was mixed with 38uM ThT. The solution was then vortexed over a 0,2, 5, 7, and 10 minute period. Controls are ThT with protein elution buffer. Results are from 3 replicates.

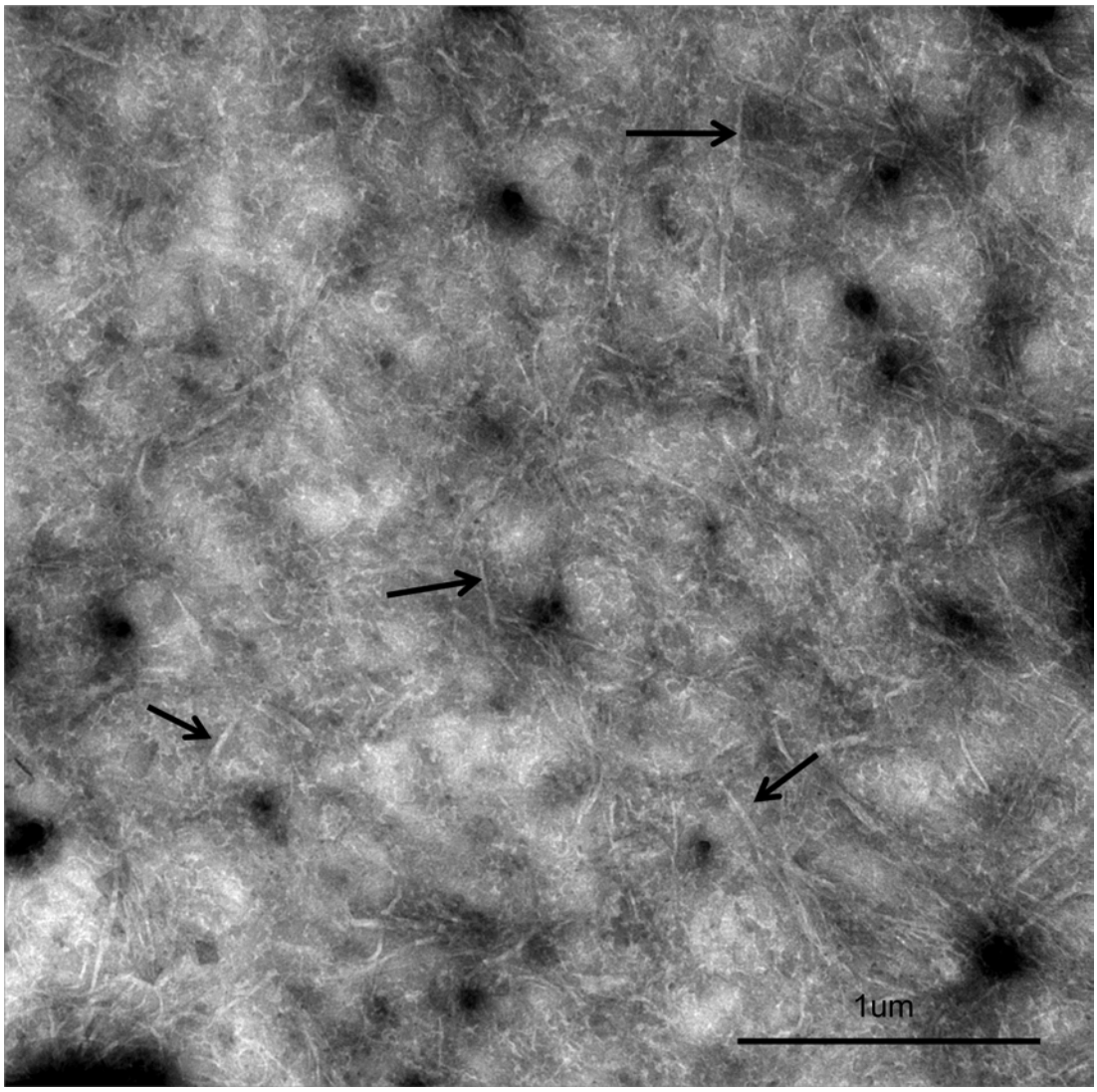

Figure 2-10. TEM micrograph of purified nHyd2 on formvar grid. 200ul of 0.18 mg/ml protein was placed on top of a formvar grid and allowed to evaporate overnight. Arrows point to striated nHyd2 filaments (L = 197 ± 52.5 nm, W = 24 ± 3.5 nm).

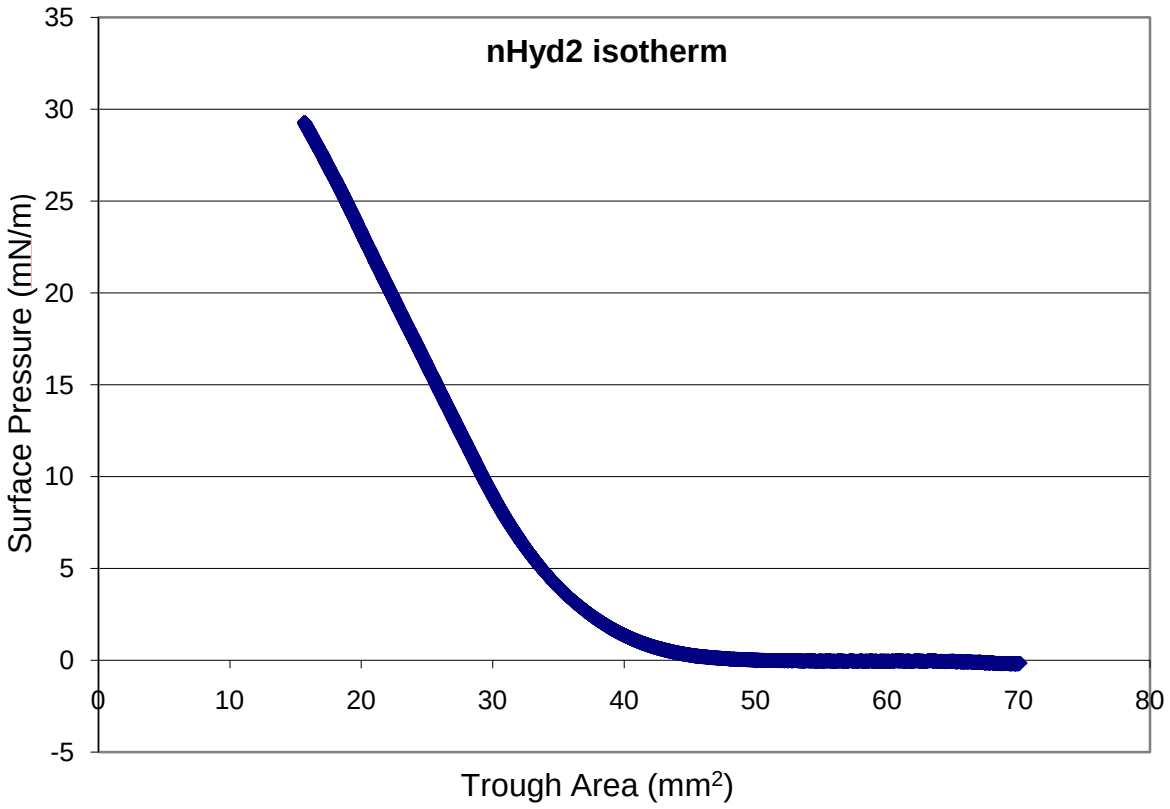

Figure 2-11. Surface pressure versus area isotherm of Hyd2 at the water-air interface at pH 6.0. Results are from 3 experiments.

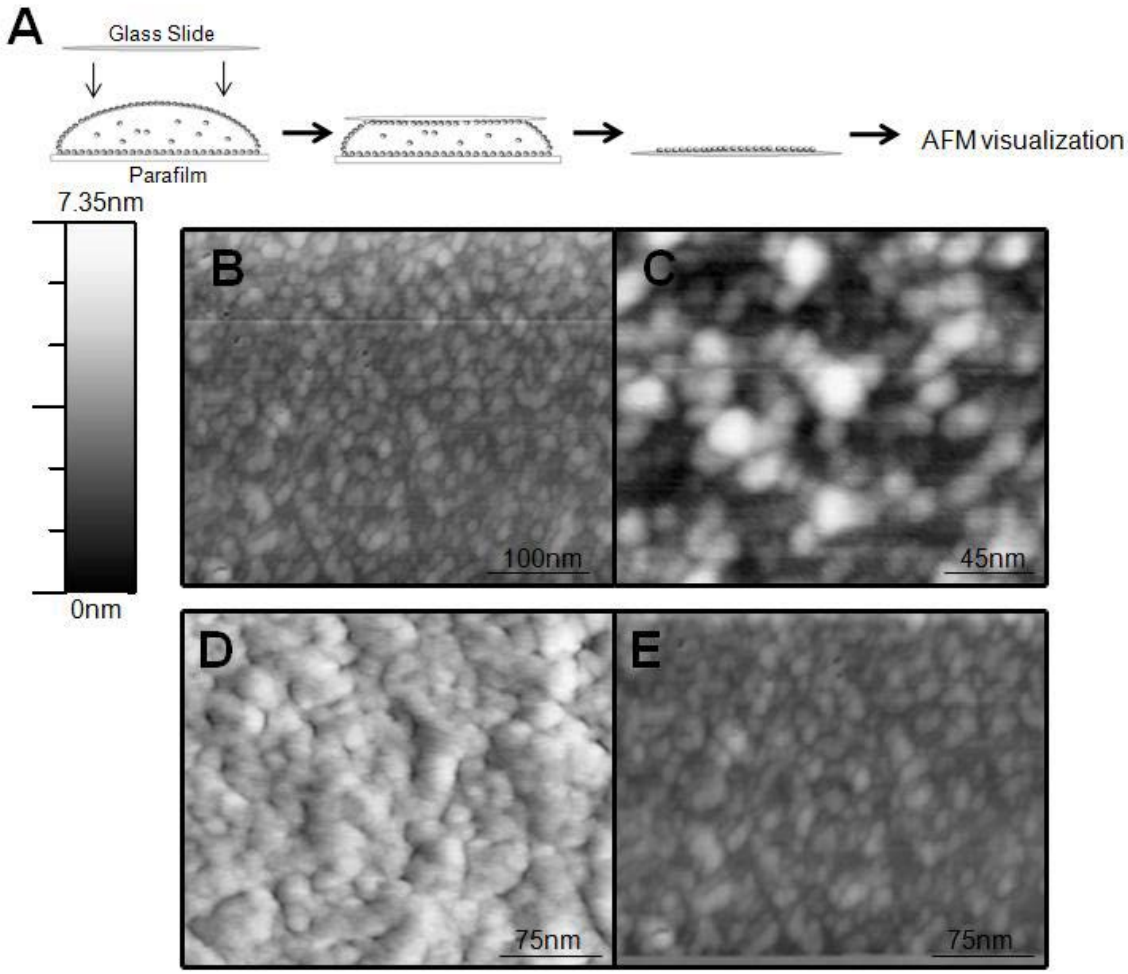

Figure 2-12. A) Schematic diagram of drop surface transfer method used to coat glass surfaces with Hyd2 protein. B) AFM height images of 12ug/ml nHyd2 coated onto a glass slide via drop surface transfer. C) High resolution image of hydrophobin monolayer. AFM micrograph of 50ul drop of 12ug/ml Hyd2 protein allowed to dry on a glass slide D) amplitude image E) height image.

Table 2-4. Contact angle of glass surface modified with recombinant Hyd2

	Control	Drop Surface Transfer	Drop Evaporation
θ_A	88.9°±0.8	56.8°±4.3	68.5°±0.9
θ_R	48.3°±6.6	17.5°±7.7	18.5°±10.8
H_20			
θ_A	75.9°±0.7	70.8°±2.9	72.9°±3.7
θ_R	44.5°±2.7	15.6°±4.4	14.5°±2.7

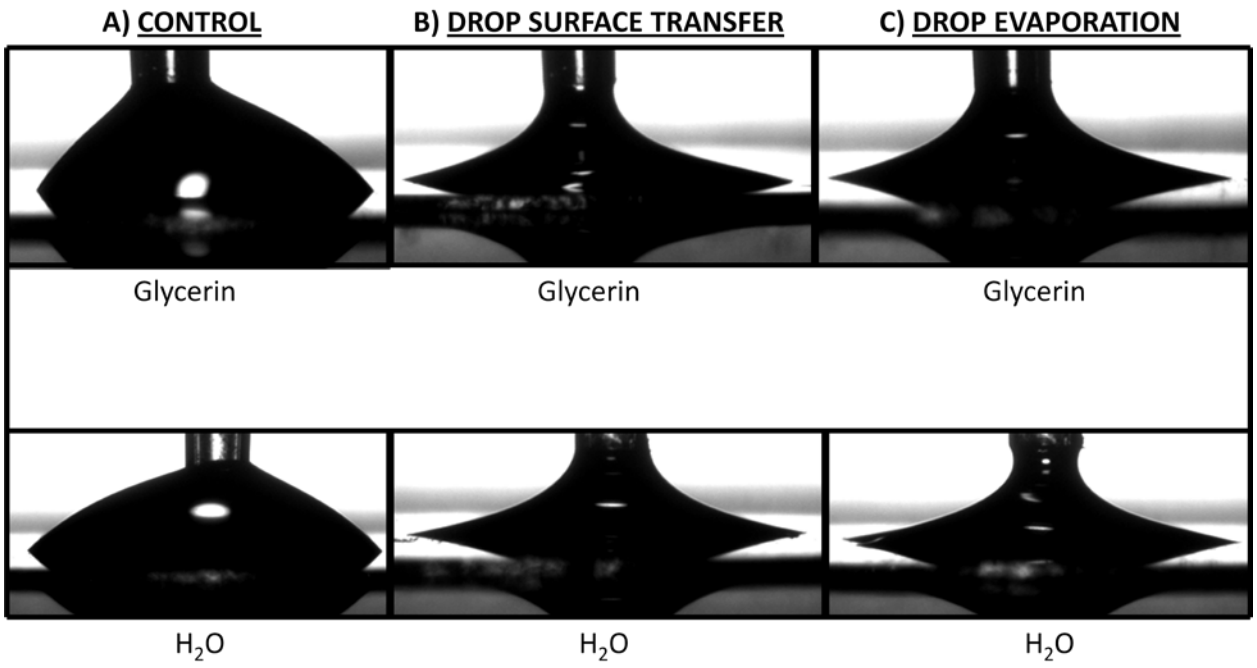

Figure 2-13. Images of receding water contact angle measurements used to determine relative change in hydrophobicity of glass surface modified with recombinant Hyd2. Droplets of Glycerin or H20 were placed on A) unmodified glass surface, B) glass surface modified by touching glass surface on top of a 100ul drop of Hyd2 solution (see drop transfer method Figure 2-16a), C) a 50ul droplet of ~130ug/ml solution placed on glass slide and allowed to evaporate overnight. All surfaces were washed 3X with H_20 to remove any unbound protein or contaminants before taking measurements.

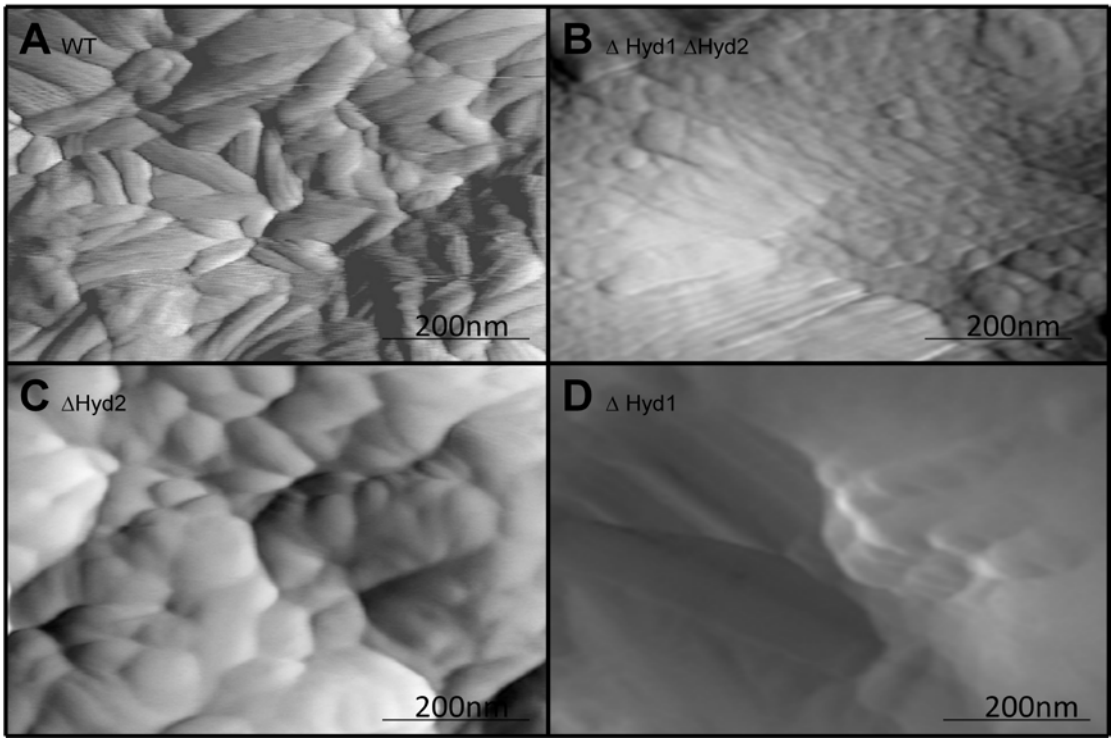

Figure 2-14. AFM surface topology of A) WT conidia B) Δhyd1Δhyd2 C) Δhyd2 D) Δhyd1

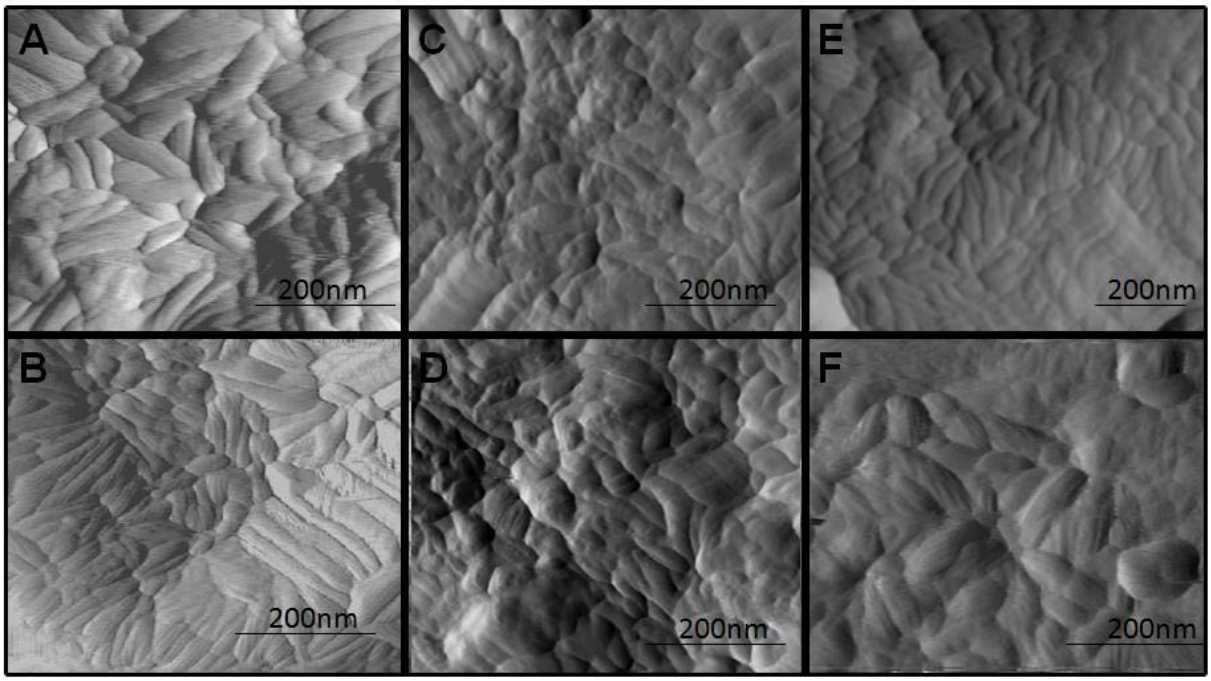

Figure 2-15. Surface phenotype of rodlet layer. A & B) WT; C& D) ΔHyd2; E & F) ΔHyd2 Trans-complemented with nHyd2.

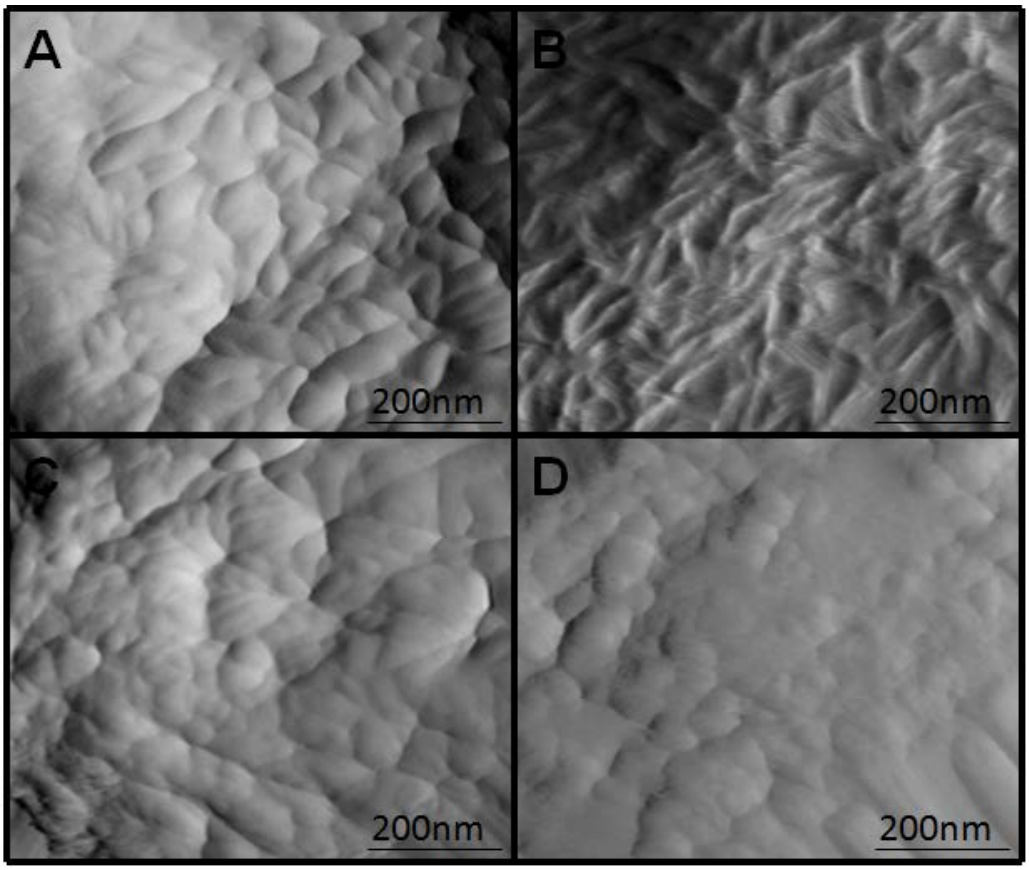

Figure 2-16. ΔHyd2 conidia Trans-complimented with nHyd2 over a 30 day time course. ΔHyd2 conidia harvested from the same PDA agar plate were trans-complemented with nHyd2 solution at A) 10 days B) 14 days C) 21 days D) 30 days.

Table 2-5. pH dependence of trans-complementation

∆hyd2 conidia	pH 4	pH 5	pH 6	pH 7	pH 8	pH10
Day 14*	-/+	-/+	+++	+	-	-
Day 30*	+	-	-	-	-	-

*Results are from 5-7 replicates

** No significant impact of pH upon nHyd2 self-assembly as determined by ThT assay

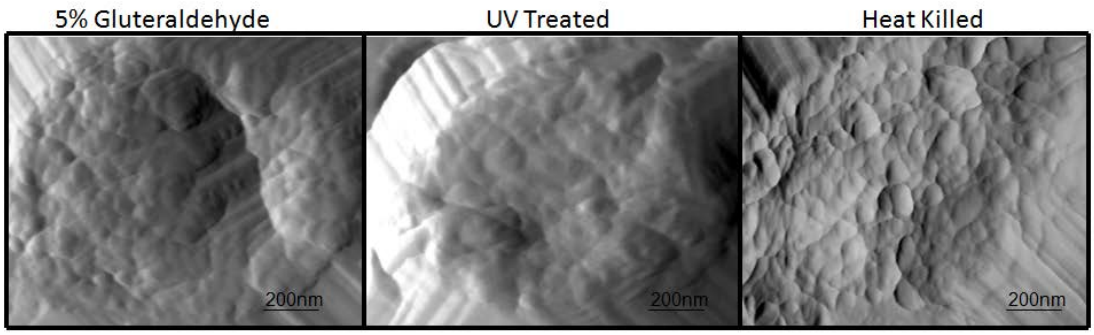

Figure 2-17. AFM micrographs of Gluteraldehyde fixed, UV treated, or Heat killed ΔHyd2 conidia that have been trans-complemented. Mutant conidia were killed with either 5% Gluteraldehyde, UV treatment, or Heat killed. Mutant conidia were then trans-complemented with ~125ug/ml recombinant Hyd2 as described in materials and methods.

APPENDIX
SURFACE MODIFICATION: ANTIMICROBIAL FILMS

Introduction

Surface Modification: Antimicrobial Films

Current research in surface modification involves improving the biocompatibility of polymers like polystyrene, polyurethane, mica, and Teflon (Anselme, 2000; Khire *et al.*, 2007). The more interesting of these investigations involves targeting of specific tissues to maximize cell-to-substrate interactions. Janssen et.al. have shown that the addition of the RGD peptide to the N-terminal domain of a class I hydrophobin,Sc3, and a truncated Sc3 (Tr-Sc3) can be used to promote fibroblast growth on to a solid surface such as Teflon(Janssen *et al.*, 2002; Scholtmeijer *et al.*, 2002). In addition to efforts aimed at enhancing the biocompatibility of synthetic materials, bacterial proliferation on polymer surfaces and subsequent host infection are important concerns regarding surface materials used in medical devices (Cho *et al.*, 2007b; Darouiche, 2004; Endo *et al.*, 1987; Smith *et al.*, 2005). The increasing resistance of bacteria to conventional antibiotics has resulted in the development of alternate strategies to fight bacterial contamination. Antimicrobial peptides offer a promising alternative to combat these problems. Cationic antimicrobial peptides (CAP's) feature several beneficial qualities such as bactericidal activity, a broad antibacterial spectra, and synergism with other antibiotics. They generally work by a non-specific interaction through permeabilization of microbial membranes and specific interactions with membrane targets (Oren *et al.*, 2007; Tossi & Sandri, 2002; Zasloff, 2002). The amino acid sequence FKVKFKVKVK is an amphipathic β-sheet CAP that was used in the design and synthesis of an antibacterial conjugate (Cho *et al.*, 2007b). In the Cho study, the antimicrobial peptide

was conjugated on to PEG-PS resin. The antibacterial, hemolytic, and antibiotic synergism effect was then tested. They concluded that this antibacterial peptide conjugate had potent antibacterial activity, no hemolytic activity, an increase in permeability of lipid membranes, and synergism with the antibiotic vancomycin. Another antimicrobial peptide CM4 is also under study for its broad bacterial susceptibility. This antimicrobial peptide is a highly cationic peptide of the cecropin family (Chen *et al.*, 2008).

It is known that the N-termini of class I and class II hydrophobins lie on the same face and are partitioned toward the more hydrophilic phase of the interfacial barrier. Also the 10-15 amino acids on the N and C termini are not crucial for the gross assembly properties of the hydrophobins (Janssen *et al.*, 2002; Kwan *et al.*, 2006). So our hypothesis is that due to the end sequence flexibility and location on the hydrophilic phase of the self-assembled monolayer, the N-termini of the hydrophobin Hyd2 from *Beauveria bassiana* can be exploited to incorporate multifunctional activity into its assembly matrix for the modification of surfaces. The inclusion of an antimicrobial peptide to a hydrophobin has yet to be described in the literature. With the advancements in intein mediated protein ligation (IPL) technology, it is now possible to couple two proteins with relative ease. IPL is a means of thioester exchange in which a protein or peptide thioester is irreversibly ligated to an N-terminal Cys-protein or peptide.

Materials and Methods

Synthesis of BK11, BK14, BK14, cysHyd2, and CM4 Plasmid Constructs

Several Hyd2 derivatives were constructed to incorporate antimicrobial activity into the self assembling properties of hydrophobins. Figure 2-3 shows the schematic

diagram of the hydrophobin derivative constructed. The antimicrobial peptide, FKVKFKVKVK was added to the N-termini, C-termini or both N & C termini of Hyd2 by primer extension PCR (Table 2-1). The forward and reverse primers included SapI and PstI restriction sites respectively for insertion into the pTWIN1 vector. Each derivative was fused to the Ssp DnaB intein for expression and purification as per the NEB protocol. Expression and inclusion body refolding was performed as previously described. The CM4 antimicrobial peptide was synthesized according to Chen et.al. 2008 with modifications. Briefly, two overlapping primer sets CM4-F1 and CM4-R1 were used to generate a recursive polymerase chain reaction product (rPCR). Next CM4-F2 and CM4-R2 was again amplified containing SapI and NdeI restriction sites at the 5' and 3' ends respectively. The cys-nHyd2 primer contained the SapI restriction site as well as the addition of three base pairs 'tgc' coding for a cysteine on the 5' end. The addition of a cysteine residue on the N-terminus of the hydrophobin allows for the intein mediated protein ligation. The cys-nHyd2 gene was first subcloned into the pDRIVE vector using Qiagen's PCR cloning kit. The cys-nHyd2 was then cut from the pDRIVE using SapI and PstI. Resulting fragment was then gel purified and ligated with the pTWIN1 vector and transformed into Top10 competent cells. Positive transformants were analyzed by PCR, enzyme digest, and DNA sequencing. The plasmid vector was then transformed into Rosetta2 (DE3) gami (Novogen) competent cells using manufacturer's protocol. Expression and purification of the cysHyd2 protein was carried our using the protocol described previously for the native Hyd2.

Results

Hyd2 Derivatives

It has been shown that the N and C-termini of class I and class II hydrophobins lie on the same face and are partitioned toward the more hydrophilic phase of the interfacial barrier (Kwan *et al.*, 2006). Also, truncations of the 10-15 amino acids on the N and C termini are not necessary for the gross assembly properties of hydrophobins. This lead to the hypothesis that short cationic antimicrobial peptides can be immobilized with a high enough degree to confer bactericidal activity without interfering in the gross self-assembly of the hydrophobin. An antimicrobial peptide, FKVKFKVKVK (FKVK) that was originally synthesized by Cho et.al 2007 was genetically added to the N and/or C termini of the class I hydrophobin Hyd2 from *Beauveria bassiana*. BK11 contains an antimicrobial peptide on the N-termini, BK14 contains the antimicrobial peptide on the C-termini and BK15 contains the antimicrobial peptide on both the N and C termini of Hyd2 (Fig. A-1). Vector constructs were sequenced by ICBR to make sure that the gene sequence is correct and in-frame for expression. Each derivative was then expressed using the method developed for the native Hyd2. Despite many attempts at intein cleavage and stepwise refolding procedures, it was discovered that we were unable to cleave off the Ssp DnaB intein (Figure A-2). The modified hydrophobin was subjected to a stepwise refolding strategy as described previously and lowered the pH to 6.0 and increased the temperature to 30^{o}C. The cleavage of the intein takes place after a shift in pH from 8.3 to 7.0 and 24 hr incubation at RT. The lowered pH and increased temperature was thought to increase cleavage efficiency. However under all conditions tested we were unable to induce cleavage of the intein from the modified Hyd2

hydrophobin (Fig. A-3). Presumably this is due to an improper folding of the derivatives which inhibited pH induced self cleavage of the intein fusion partner.

Intein Mediated Protein Ligation

To continue testing the hypothesis that short cationic antimicrobial peptides can be immobilized in a Hyd2 monolayer a new strategy was developed. Intein mediated protein ligation allows the ligation of a protein with an N-terminal cysteine residue and a peptide with a C-terminal thioester forming a native peptide bond (New England Biolabs). To accomplish this goal the Hyd2 protein had to be modified to contain a cysteine on its N-Terminal. An antimicrobial peptide with a C-Terminal thioester was also generated by fusion to an intein (Fig. A-4). The antimicrobial peptide CM4 was expressed in a pTWIN1 vector with an Mxe GyrA intein which will generate a thioester on the C-terminus. The presence of the CM4 in the vector was confirmed by PCR and sequencing. The hydrophobin is generated with a cysteine residue on the N-terminus. The antimicrobial peptide and Hyd2 are then mixed to produce a peptide bond generating a CM4-Hyd2 fusion (Fig A-5).

Discussion

Hyd2 Surface Modification; Antimicrobial Films

Our initial attempts to produce Hyd2 derivatives have thus far been unsuccessful. Several Hyd2 derivatives were constructed, BK11, BK14, and BK15 consisting of an N-terminal, C-Terminal, and N&C-terminal antimicrobial peptide FKVKFKVKVK by addition to the 5' or 3' ends of the cDNA sequence. Each derivative was cloned into the pTWIN1 vector and transformed into the Rosetta2 (DE3) strain of *E.coli*. The derivative Hyd2 proteins were then expressed; however the intein moiety that was attached to the N-terminus for rapid purification was unable to be cleaved. Despite multiple experiments

to refold and optimize the cleavage reaction, we were unable to remove the intein fusion partner from each derivative. We conclude that the protein was unable to fold properly and thus the unable to induce cleavage.

To overcome this obstacle, we developed a different strategy for generation hydrophobin derivatives. We decided to generate the derivatives by way of intein mediated protein ligation (IPL). Using IPL, it is possible to allow the hydrophobin to self assemble on to the surface first and then couple another protein or peptide on to the monolayer. This reduces the risk of steric hindrance inhibiting self assembly and allows for designer peptide modification. However, we have been unsuccessful thus far, but confident that continued work will result in the desired modifications. The applications for this become numerous from antimicrobial, antifungal, cell adhesion, and enzyme immobilization.

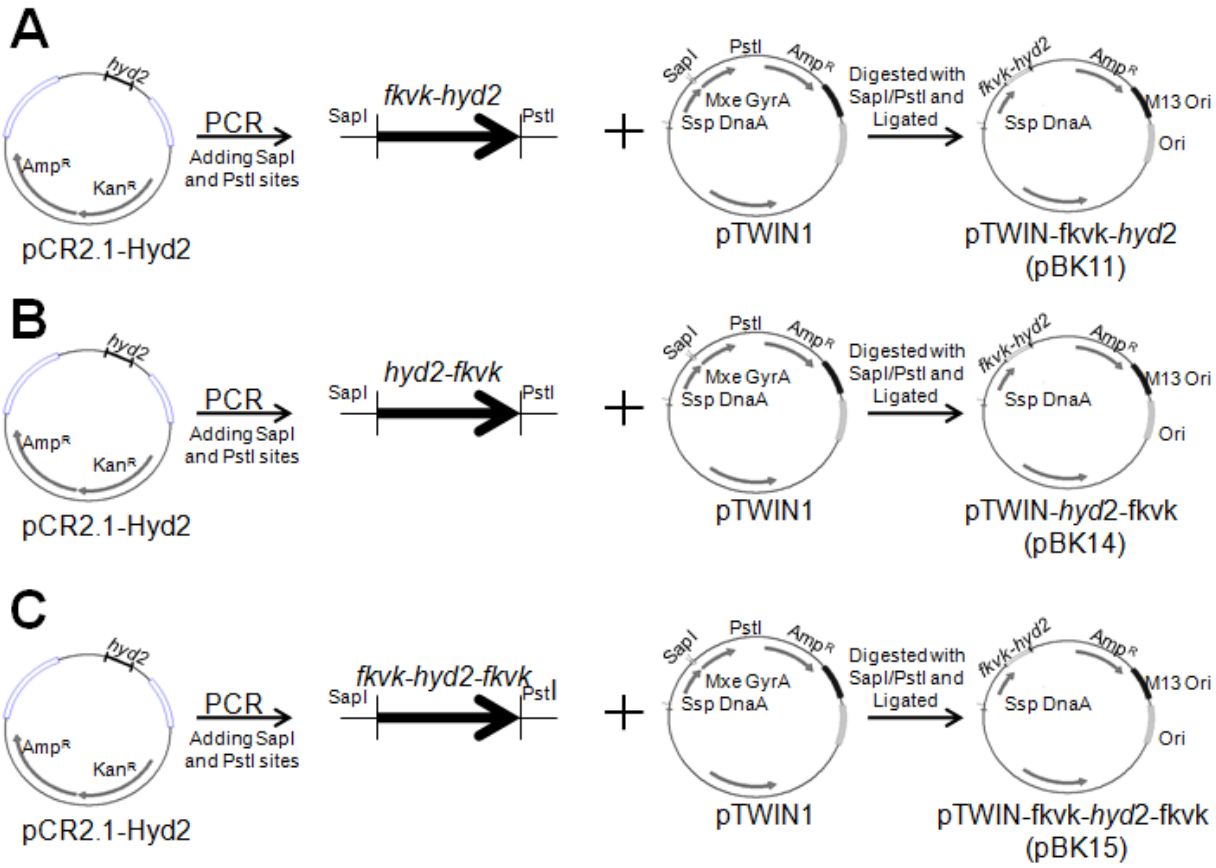

Figure A-1. Vector construction of Hyd2 derivatives. Vector construction of hyd2 with A) a N-terminal FKVKFKVKVK antimicrobial peptide, B) a C-Terminal FKVKFKVKVK antimicrobial peptide, C) a N and C-terminal FKVKFKVKVK antimicrobial peptide.

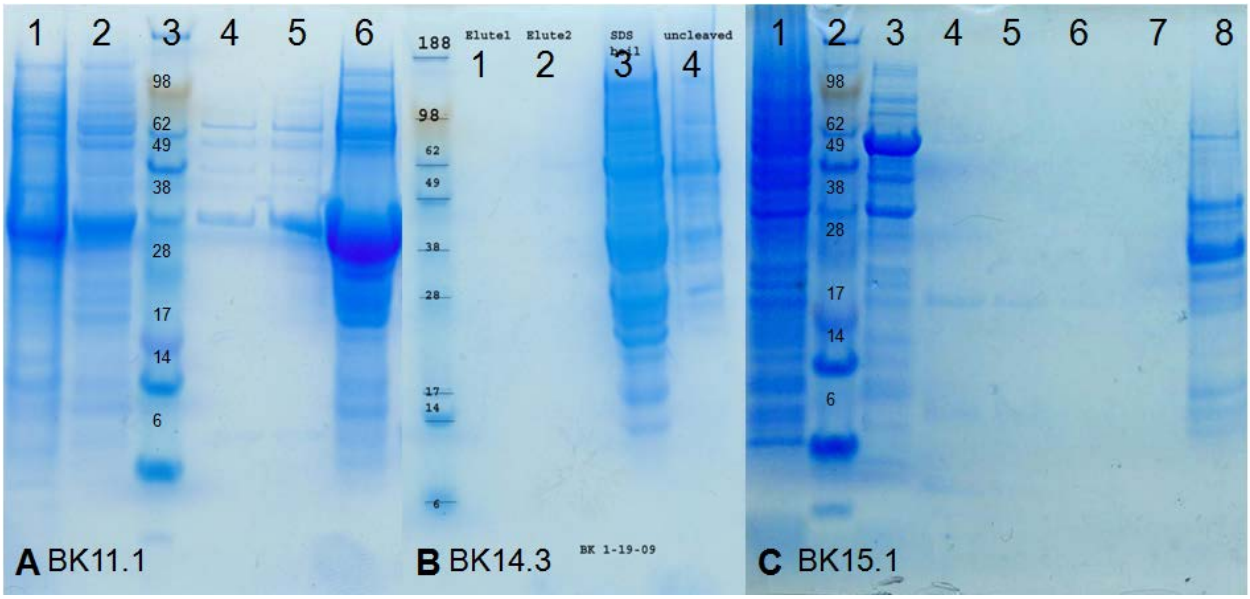

Figure A-2. SDS PAGE gels of Hyd2-antimicrobial derivates. A) BK11, refolded protein product, lane 1; flowthrough after binding to chitin bead column , lane 2; protein elution after intein cleavage reaction lane 4 and 5; fraction after boiling chitin bead in SDS, lane 6. B) BK14, elute after intein cleavage reaction, lane 1 and 2; fraction after boiling chitin beads in SDS. C) BK14 refolded protein product, lane 1; flowthough after binding to chitin beads, lane 3; elute after intein cleavage reaction lanes 4-7; fraction after boiling chitin beads in SDS.

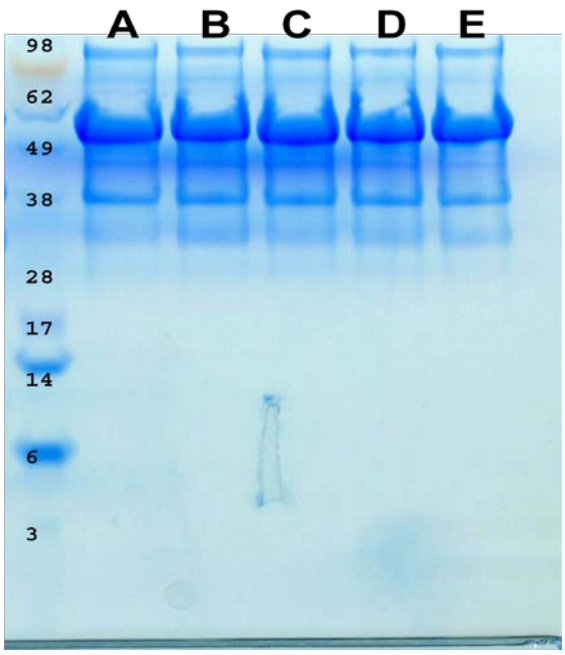

Figure A-3. Cleavage optimization experiment on BK11.1. A)24hr, RT, pH 7.0 B) 24hr, RT, pH 6.0 C) 24hr, 30^{o}C, pH 7.0 D) 24hr, 30^{o}C, pH 6.0 E) 24hr, 16^{o}C, pH 6.0.

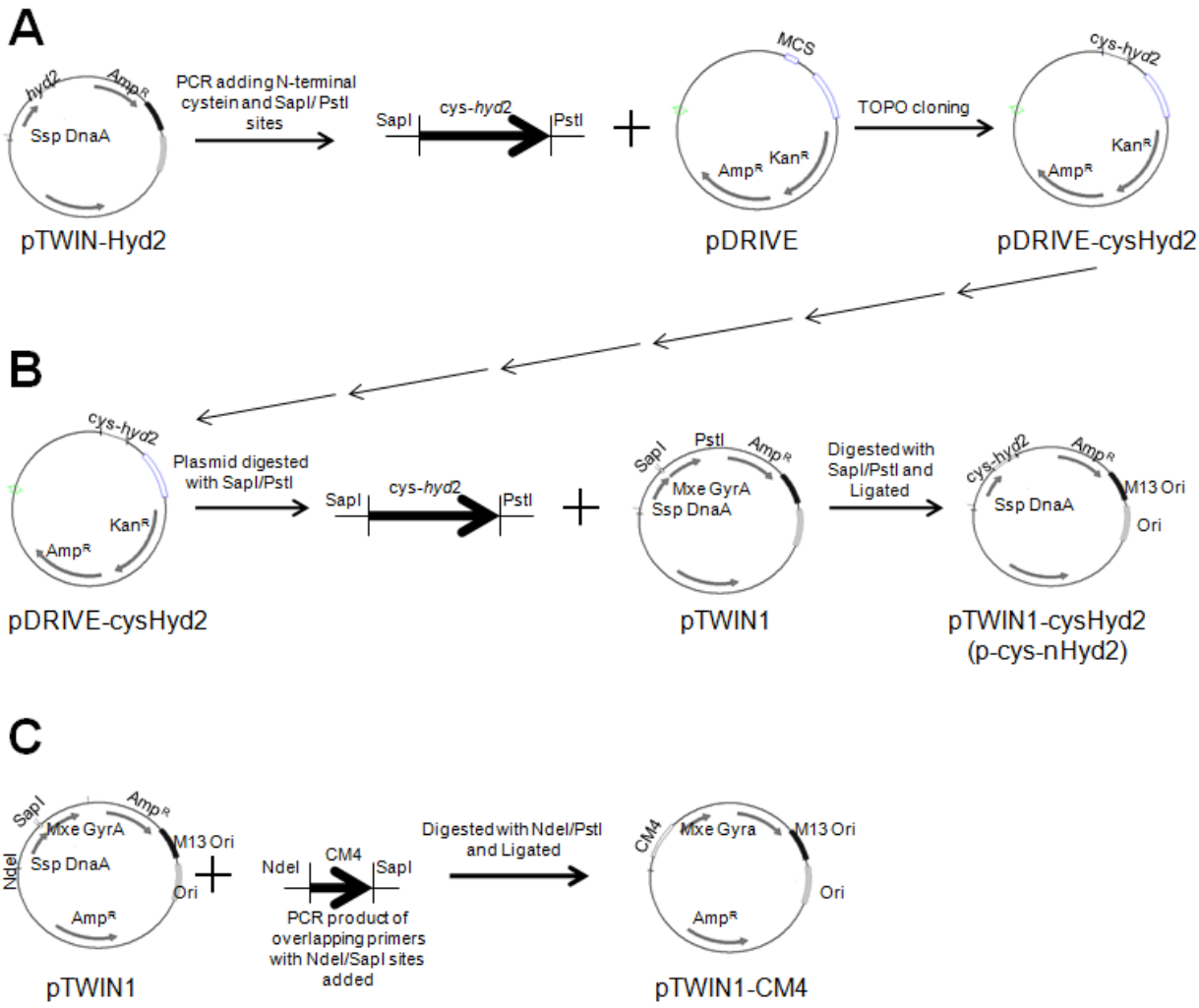

Figure A-4. Vector construction of A) cys-Hyd2 with N-terminal intein and B) CM4 with C-Terminal Intein.

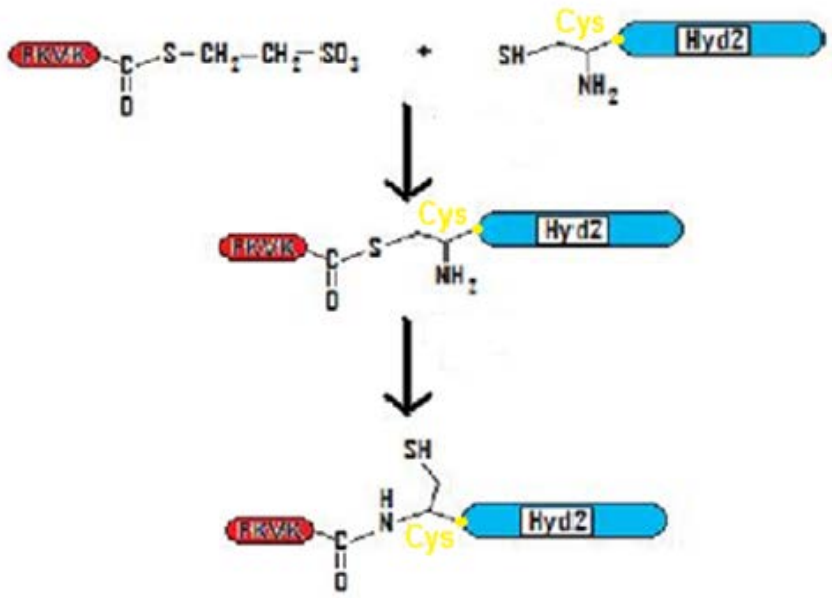

Figure A-5. IPL reaction of Antimicrobial peptide with Hyd2 for surface modification.

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BIOGRAPHICAL SKETCH

Brett Kirkland grew up under the watchful eyes of Fred and Sandra Kirkland. He grew up enjoying baseball, fishing, and camping. As a youth he discovered that he had an interest in the sciences. Brett started his undergraduate career in the fall of 1997 at the University of Florida. In order to put himself through college he began working a local hospital where he worked in the microbiology department. Eventually he began to want more out of his degree that just performing protocol microbiology. He began seeking a means for advancing his education that would lead to a career in science. During the early spring of 2004 he was given the opportunity to volunteer in a laboratory in the Department of Microbiology and Cell Science where he soon realized that research was the direction he wanted to pursue. Brett became a graduate student in the Department of Microbiology and Cell Science in the fall of 2005, where he will earn a PhD from the University of Florida. Brett is the first in his family to earn a PhD and would like to thank everyone who helped make it possible.