ADHESION PROPERTIES AND CELL SURFACE CHARACTERISTICS
OF THE ENTOMOPATHOGENIC FUNGUS Beauveria bassiana: A LINK BETWEEN
MORPHOLOGY AND VIRULENCE

By

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2005
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by

Diane J. Holder
This document is dedicated to my parents, my sister and her children
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I would like to thank my family for always being there for me, and my major advisor, Dr. Nemat Keyhani, without whom this would all have been impossible. I would also like to thank all the members of Dr. Keyhani’s lab (past and present) for the help, support and friendship that I will always treasure. I also would like to recognize the other members of the department that helped me with both hands on and theoretical applications, especially Waultraud Dunn, Donna Williams, Dr. Henry Aldridge, Louise Monroe, members of Dr. James Preston’s lab, Dr. Keelnatham Shammungam’s lab, and Dr. Julie Maupin’s lab. I would also like to acknowledge the students, staff and professors in PERC, for their help and the use of their equipment.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>2</td>
</tr>
<tr>
<td>Biological Control</td>
<td>7</td>
</tr>
<tr>
<td>Fungal Biocontrol Agents</td>
<td>7</td>
</tr>
<tr>
<td>Beauveria bassiana as a Biocontrol Agent</td>
<td>7</td>
</tr>
<tr>
<td>Factors Affecting Adhesion</td>
<td>8</td>
</tr>
<tr>
<td>Specific Binding</td>
<td>9</td>
</tr>
<tr>
<td>Nonspecific Binding</td>
<td>9</td>
</tr>
<tr>
<td>Steric, Bridging and Depletion forces</td>
<td>10</td>
</tr>
<tr>
<td>Hydrophobic Interactions</td>
<td>11</td>
</tr>
<tr>
<td>Specific Molecules or Structures Involved in Fungal Adhesion</td>
<td>12</td>
</tr>
<tr>
<td>Molecular Biology</td>
<td>14</td>
</tr>
<tr>
<td>Methods Used to Study Global Differential Gene Expression in Fungi</td>
<td>14</td>
</tr>
<tr>
<td>Differential screening of cDNA libraries</td>
<td>15</td>
</tr>
<tr>
<td>Real Time RT-PCR</td>
<td>17</td>
</tr>
<tr>
<td>Objectives</td>
<td>18</td>
</tr>
<tr>
<td>2 ADHESION OF THE ENTOMOPATHOGENIC FUNGUS BEAUVERIA BASSIANA TO SUBSTRATA</td>
<td>20</td>
</tr>
<tr>
<td>Introduction</td>
<td>20</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>21</td>
</tr>
<tr>
<td>Cultivation of Fungi</td>
<td>21</td>
</tr>
<tr>
<td>FITC-labeling of B. bassiana Cells</td>
<td>22</td>
</tr>
<tr>
<td>Adhesion Assay</td>
<td>22</td>
</tr>
<tr>
<td>Enzyme Treatments</td>
<td>23</td>
</tr>
<tr>
<td>Effect of pH Influence on Attachment</td>
<td>24</td>
</tr>
</tbody>
</table>
3 SURFACE CHARACTERISTICS OF THE ENTOMOPATHOGEN
BEAUVERIA BASSIANA ....................................................................................38

Introduction.................................................................................................................38
Materials and Methods ...............................................................................................43
  Atomic Force Microscopy ...................................................................................44
  Zeta Potential .......................................................................................................44
  MATH Assay .......................................................................................................44
  Contact Angle Determination ............................................................................45
Results.........................................................................................................................45
  Atomic Force Microscopy ...................................................................................45
  Zeta Potential .......................................................................................................46
  MATH Assay .......................................................................................................46
  Contact Angles ....................................................................................................48
Discussion...................................................................................................................49

4 SUPPRESSIVE SUBTRACTION HYBRIDIZATION ANALYSIS OF
BEAUVERIA BASSIANA GROWN ON INSECT CUTICLE ................................55

Introduction.................................................................................................................55
Virulence Factors........................................................................................................55
Adherence and Colonization factors .......................................................................56
Toxins..................................................................................................................57
Molecules Involved in Evading Host Defences ..................................................58
Siderophores........................................................................................................58
Molecules Involved in Toxin Transport ..............................................................58
Suppression Subtractive Hybridization ...............................................................59
Materials and methods................................................................................................60
Cultivation of Fungi ............................................................................................60
  Cells grown in the presence of glucose (source of driver RNA)..................60
  Cells grown on insect cuticle (source of tester RNA)..............................60
RNA Isolation......................................................................................................61
Suppression Subtractive Hybridization .................................................................61
Results.........................................................................................................................61
Discussion...................................................................................................................65

5 MOLECULAR ANALYSIS OF TWO BEAUVERIA BASSIANA
HYDROPHOBINS AND A HYDROPHOBIN LIKE PROTEIN .................70

Introduction.................................................................................................................70
Materials and Methods ...............................................................................................73
Cultivation of Fungi ...........................................................................................73
RNA Isolation......................................................................................................74
Rodlet Layer Extraction ......................................................................................74
Mass Peptide Spectrometry (Peptide Fingerprinting) .........................................75
Reverse Transcriptase RT-PCR...........................................................................76
Results.........................................................................................................................77
Identification of a \textit{B. bassiana} Cell Wall Hydrophobin ........................................77
Reverse Transcriptase RT-PCR...........................................................................77
Discussion...................................................................................................................80

6 GENERAL DISCUSSION .........................................................................................83
Statement of Hypotheses ............................................................................................83
Is There a Measurable Difference in The Cell Surface Characteristics of The Single Cell Propagules of \textit{B. bassiana}? .................................................................83
Visual Differences ..................................................................................................83
Differences in Hydrophobicity .............................................................................83
Differences in Effective Surface Charge .........................................................84
Cell Wall Proteins.................................................................................................85
Conclusions .............................................................................................................86
Are there Differences in the Binding Properties of These Propagules, Which Can Be Related to the Cell Surface Characteristics? ....................................................86
Adhesion Profiles of Aerial Conidia, Submerged conidia and Blastospores .........86
Conclusion.............................................................................................................86

APPENDIX AGROBACTERIUM MEDIATED TRANSFORMATION OF BEAUVERIA BASSIANA ........................................................................................88
Introduction.................................................................................................................88
Materials and Methods ...............................................................................................89
Fungal Cultures ........................................................................................................89
\textit{Agrobacterium tumefaciens} Cultivation.................................................................90
Transformation Procedure ...................................................................................90
Discussion...................................................................................................................93

LIST OF REFERENCES.................................................................................................96

BIOGRAPHICAL SKETCH ...........................................................................................105
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Classification <em>B. bassiana</em></td>
</tr>
<tr>
<td>2-1</td>
<td>Effects of various competitors and chemicals on <em>B. bassiana</em> cell-type adhesion</td>
</tr>
<tr>
<td>2-2</td>
<td>Effect of pH on <em>B. bassiana</em> cell-type adhesion</td>
</tr>
<tr>
<td>2-3</td>
<td>Effects of various enzymatic treatments on <em>B. bassiana</em> cell-type adhesion</td>
</tr>
<tr>
<td>3-1</td>
<td>Contact angle values for the three cell types for water, bromonapthalene and glycerol with calculated interfacial and polar free energy values</td>
</tr>
<tr>
<td>3-2</td>
<td>Advancing and receding water contact angle data with calculated surface energy values for three <em>B. bassiana</em> single cell propagules</td>
</tr>
<tr>
<td>4-1</td>
<td>Blast hits of sequence fragments from the SSH library to virulence factors and allergens</td>
</tr>
<tr>
<td>5-1</td>
<td>Primer sequences and product size for the reverse transcriptase RT-PCR</td>
</tr>
<tr>
<td>5-2</td>
<td>mRNA abundance of <em>bhd1, bhd2</em> in <em>Beauveria bassiana</em> single cell propagules</td>
</tr>
<tr>
<td>A-1</td>
<td>Putative transformants obtained from <em>Agrobacterium tumefaciens</em> mediated transformation of <em>B. bassiana</em> with selection markers for hygromycin B (<em>hph</em>) and neomycin (<em>neo</em>) resistance</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>The life-cycle of a typical hyphomycete entomopathogen.</td>
<td>6</td>
</tr>
<tr>
<td>2-1</td>
<td>Bright field and fluorescent microscopy of FITC-labeled <em>B. bassiana</em> aerial conidia, blastospores and submerged conidia</td>
<td>25</td>
</tr>
<tr>
<td>2-2</td>
<td>Fluorescent intensity of FITC labeled aerial conidia, blastospores, and submerged conidia as a function of cell number</td>
<td>27</td>
</tr>
<tr>
<td>2-3</td>
<td>Adhesion of aerial conidia to glass and silinated glass surfaces.</td>
<td>27</td>
</tr>
<tr>
<td>2-4</td>
<td>Adhesion of blastospores to glass and silinated glass surfaces.</td>
<td>28</td>
</tr>
<tr>
<td>2-5</td>
<td>Adhesion of submerged conidia to glass and silinated glass</td>
<td>28</td>
</tr>
<tr>
<td>2-6</td>
<td>Saturation point of binding sites for <em>B. bassiana</em> aerial conidia, blastospores, and submerged conidia to microtiter plates</td>
<td>29</td>
</tr>
<tr>
<td>2-7</td>
<td>Quantitative adhesion and influence of washing on adhesion of aerial conidia, blastospores, and submerged conidia to silinated F-200 (hydrophobic), F-200 (weakly polar), and F-600 (hydrophilic) microtiter plates</td>
<td>30</td>
</tr>
<tr>
<td>3-1</td>
<td>Contact angles (θ) are formed at the interface between the liquid, solid and gas boundaries of a droplet on the surface of interest.</td>
<td>40</td>
</tr>
<tr>
<td>3-2</td>
<td>Atomic force micrographs of <em>B. bassiana</em> conidia, submerged conidia and blastospores</td>
<td>46</td>
</tr>
<tr>
<td>3-3</td>
<td>Zeta potential values for the three spore types as a function of pH.</td>
<td>47</td>
</tr>
<tr>
<td>3-4</td>
<td>Microbical adhesion to hydrocarbon and hydrophobicity indices for <em>B. bassiana</em> aerial conidia, blastospores and submerged conidia.</td>
<td>47</td>
</tr>
<tr>
<td>4-1</td>
<td>Relative numbers of gene fragments representing functional groups present in the subtracted library</td>
<td>62</td>
</tr>
<tr>
<td>4-2</td>
<td><em>B. bassiana</em> H1 hydrophobin genomic sequence</td>
<td>66</td>
</tr>
<tr>
<td>4-3</td>
<td>Alignment of H1 and H2 with homologous hydrophobins</td>
<td>67</td>
</tr>
</tbody>
</table>
4-4 Bootstrapped (1000) phylogenetic tree of class I and class II hydrophobins........68

5-1 SDS –PAGE (10% polyacrylamide, Bis-Tris) Gel of SDS soluble/TFA insoluble cell wall proteins. .................................................................77

5-2 Mass spectroscopy data showing the mass of individual amino acids in one of the two main fragments from the 12 KDa trifluoroacetic acid soluble/sodium dodecyl sulfate insoluble B. bassiana cell wall protein. ........................................79

5-3 Mass peptide fingerprinting results for two identifiable fragments of the 12 KDa trifluoroacetic acid soluble, sodium dodecyl sulfate insoluble B. bassiana cell wall protein........................................................................................................80

5-4 Comparison of Bhd1 and Bhd2 and other hydrophobins consensus spacing for Class I and Class II hydrophobins................................................81
Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

ADHESION PROPERTIES AND CELL SURFACE CHARACTERISTICS OF THE ENTOMOPATHOGENIC FUNGUS Beauveria bassiana: A LINK BETWEEN MORPHOLOGY AND VIRULENCE

By Diane J. Holder

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Chair: Nemat O. Keyhani
Major Department: Microbiology and Cell Science

The entomopathogen Beauveria bassiana produces three distinct in vitro, single cell propagules: aerial conidia, blastospores and submerged conidia. Atomic force microscopy (AFM) was used to visualize the surface of aerial conidia and confirmed the presence of a rodlet layer that was absent from the surface of both blastospores, which were smooth, and submerged conidia, which appeared coarse. Interfacial free energies of interaction and hydrophobicity indicies, derived from contact angle data, and hydrocarbon partitioning revealed differential properties of the three propagules regarding cell surface hydrophobicity ranging from strongly hydrophobic (aerial conidia) to hydrophilic (blastospores). Adhesion studies with fluorescently labeled cells suggested that 1) aerial conidia bound better to hydrophobic surfaces, 2) blastospores bound better to hydrophilic surfaces, and 3) submerged conidia bound equally well to both types of surfaces.
The effective surface charge (zeta potential) of the three single cell propagules was predominantly positive at low pH (pH 3-4) decreasing to negative values at higher pH values (pH 6-8). Aerial conidial surface charge varied the most with respect to pH (+22 mV to -47 mV), while submerged conidia varied moderately (+7 mV to – 13.4 mV) and the blastospores showed minor variation (+3.2 mV to – 4.65 mV). The gene for a beaverial hydrophobin (bhd1) was identified from a suppression-subtracted library generated from cells grown in the presence of insect cuticle as opposed to glucose. Real-time, reverse transcriptase PCR of two Beauveria bassiana specific hydrophobins and a hydrophobin like protein (bhd1, bhd2 and bsn) showed that bhd1 mRNA levels were relatively high in most cell types analyzed, with the highest abundance in submerged conidia. Transcript for bhd2 was detected primarily in aerial conidia and submerged conidia, whereas transcript for bsn was not detected under the conditions tested. A SDS insoluble/TFA soluble constituent of the cell wall of B. bassiana conidia was identified as bhd2 by peptide mass fingerprinting.
CHAPTER 1
LITERATURE REVIEW

The earliest reports of a fungal entomopathogen, possibly the organism that would come to be known as *Beauveria bassiana* (Balsamo) Vuillemin, came from China, as far back as 2700 BC (79). It was not until 1835 that Agostino Bassi demonstrated that Calcino, or White Muscardine, a disease that was devastating the Italian silkworm industry, was contagious and caused by a parasitic fungus (63). Balsamo Crivelli officially named the organism *Botrytis paradoxica*, eventually changing the name to *Botrytis bassiana* to honor the man who first described it.

In 1912, Vuillemin, determined that there were enough features peculiar to *Botrytis bassiana* to assign it to the new genus *Beauveria* (19). There now are multiple species in the genus *Beauveria* Vuill. some of the most important ones are: *B. bassiana*, *B. brongniartii*, and *B. alba*. *B. bassiana* and *B. brongniartii* well known entomopathogens with a wide host range, including arthropods other than insects, are now being used as biological control agents to control a variety of crop damaging insects. *B. alba* is mainly isolated as an indoor contaminant and displays the lowest pathogenicity of these three *beauveria* species (1). Due to the practical applications of fungal entomopathogens as biological control agents, the biology (and to a lesser extent the molecular biology) of these fungi has been the subject of much research.

Major efforts have been targeted towards isolation and characterization of strains with high virulence, improved cost effectiveness, and to technologies that could be applied to other economically important *Ascomycetes*. One of the most important steps in
the host-pathogen interaction is the initial attachment of the fungus to the host cuticle. Modifying the formulation of commercial products, or of the fungus itself, namely to improve targeting and attachment to the host cuticle, may lead to improvements in infection rates, and host mortality, and hence the effectiveness of the biocontrol.

This dissertation examines the physiological and molecular determinants of *B. bassiana* attachment. New techniques to evaluate these adhesion profiles were developed, and a protein implicated in the adhesion process were isolated and characterized.

**Beauveria bassiana**

All fungal phyla include species that are able to reproduce either sexually or asexually. The production of multiple spore types increases the chances of survival during adverse environmental conditions (1). These spore types can be produced in response to environmental conditions, as well as at different times in the lifecycle and can have different dispersal mechanisms.

In 2002, Huang et al. (47) identified *Cordyceps bassiana* as the ascomycote teleomorph of *B. bassiana*. However, the organism is most frequently described and identified in the anamorph stage and assigned to the Deuteromycota. Taxonomical identification within the Deuteromycota relies heavily on physical characteristics such as shape, size and color, as well as the manner in which the asexual spores, or conidia, are produced.

It is a common, and often useful, practice to use separate nomenclatures for different stages of the same species (Table 1-1), because most Ascomycetes produce different spore types specific to the particular stage in the life cycle in which they find themselves and in some species the sexual stage may occur as infrequently as once a year
Predominant stages tend to be the most commonly described (the descriptions are usually based morphological characteristics such as spore types); as a result connections between different lifecycle stages within the same fungus are not always immediately apparent. Fungi for which a sexual stage has not yet been identified are considered second (deutero) class, imperfect fungi, and are currently assigned to the artificially constructed phylum/class. Deuteromycota/Deuteromycetes (1).

Correct nomenclature involves assigning the fungus a holomorphic name once the sexual and asexual stages have been officially linked and the organism is considered “complete.” The whole organism, with all life cycles, is the holomorph; the sexual stage, the teleomorph, and the asexual stage, the anamorph

Table 1-1. Classification B. bassiana

<table>
<thead>
<tr>
<th>Holomorph</th>
<th>Anamorph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Fungi</td>
</tr>
<tr>
<td>Phylum</td>
<td>Ascomycotina</td>
</tr>
<tr>
<td>Subphylum</td>
<td>Pezizomycotina</td>
</tr>
<tr>
<td>Class</td>
<td>Sordariomycetes</td>
</tr>
<tr>
<td>Subclass</td>
<td>Hypocreomycetidae</td>
</tr>
<tr>
<td>Order</td>
<td>Hypocreales</td>
</tr>
<tr>
<td>Family</td>
<td>Clavicipitaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Cordyceps</td>
</tr>
<tr>
<td>Species</td>
<td>bassiana</td>
</tr>
</tbody>
</table>

1 National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD

Species within the genus Beauveria are typically differentiated from other fungi by morphological characteristics. They are filamentous fungi that produce colorless (hyaline) aerial conidia from conidiogenous cells freely on the mycelia. This characteristic places them within the moniliaceous (having hyaline conidia) Hyphomycetes (19). Aerial conidia are initially produced as terminal swellings formed on the neck of the conidiophore. The next conidium grows laterally, half way up the first neck of the conidiophore, in another direction, and is pushed upwards by sympodial
growth (19). The resulting denticulate rachis, with denticles equally wide as the rachis, is characteristic of Beauveria spp.

*B. bassiana* colonies grow relatively slowly and can appear powdery or wooly, with colors ranging from white to yellow, and occasionally pinkish. Aerial hyphae are septate, smooth, hyaline, and about 2 µm wide. Submerged hyphae are similarly structured, but larger (1.5 – 3µm) (19). Conidiogenous cells, which arise from short swollen stalk cells, are often found in dense clusters or whorls. They consist of a globose base and the characteristic denticulate rachis. The aerial conidia are hyaline, smooth, relatively thin walled and vary from being oval to spherical depending on the species, and occasionally by cultural conditions (19, 47).

Typical of hyphomycete entomopathogens, *B. bassiana* invades through the host cuticle, although as with other hyphomycetes, entry through the digestive tract is also possible. The initial and crucial steps in the infection process are attachment to, and penetration of, the host cuticle. Arthropod cuticles are complex structures, which in the case of insects are composed of two main layers the epicuticle and the procuticle.

The epicuticle, a thin layer which overlays the procuticle, lacks chitin, but is composed of sklerotinized proteins overlaid by a waxy layer containing fatty acids, sterols, and lipids. The bulk of the cuticle, the procuticle, consists of chitin embedded in a protein matrix (15, 40). Fungal entomopathogens use mechanical pressure and a mixture of enzymes to penetrate and dissolve the insect cuticle (15). Although several entomopathogens use swellings at the tip of the germ tube (appressoria) to generate mechanical pressure and increase attachment to the insect cuticle, such structures are rarely observed in *B. bassiana*. However, the battery of enzymes including proteases,
and chitinases produced by this entomopathogen are similar in nature to those produced by other hyphomycete entomopathogens such as *Metharrhizium anisiopliae* (6, 77).

Once the fungal hyphae reach the hemocoel, thin walled, yeast like, hyphal-bodies, or blastospores, are generated and dispersed throughout the host (40). Host death appears to result from a number of factors including production of toxins by the fungus, physical obstruction of the circulatory system, invasion of organs, and nutrient depletion.

Upon host death, the parasite switches from yeast-like to hyphal growth invading all the tissues of the host body, while attempting to reduce or eliminate competing organisms with a variety of antimicrobial metabolites. The mummified corpse can remain in the environment unchanged for months, but under favorable conditions the hyphae emerge from within the corpse, sporulate and the resulting aerial conidia are dispersed via air or water (40).

*Beauveria* sp. produce a number of metabolites some of which have cytotoxic effects alexopoulos (1). These metabolites include beauvericin, bassianolide, beauveriolides, bassianin, tenellin, and oosporein. Beauvericin and bassioanolide are ionophores that differ in specificity for cations. Beauvericin, a hexadepsipeptide, has antimicrobial activity against both gram-negative and gram-positive bacteria, is toxic to brine shrimp with a LD$_{50}$ of 2.8 μg ml$^{-1}$ water, but has no demonstrated insecticidal effects (81). Bassianolide, a cyclo-octadepsipeptide, also has antimicrobial effects and is lethal to silkworm larvae at a concentration of 13 ppm (81).

Although beauveriolides are structurally related to beauvericin and bassioanolide, they are not as well characterized, and their antimicrobial or insecticidal potential have yet to be described (81). Namatame et al. (2004) have recently shown that beauveriolides
have an inhibitory effect on lipid drop formation in mouse erythrocytes and as a result could be marketed as anti-cholesterol drugs. According to their data, beauveriolides have few cytotoxic effects on mouse cells at levels up to 100 mg\(^{-1}\) day\(^{-1}\).

The pigments, bassianin, tenellin, and oosporein are toxic to erythrocyte membrane ATPases (50). Oosporein is also a denaturing agent and a potent antibiotic specific to gram-positive organisms. The toxicity of these pigments towards insect host cells has not been well defined (81).

![Diagram of the life cycle of a typical hyphomycete entomopathogen](image)

**Fig. 1-1.** The life-cycle of a typical hyphomycete entomopathogen. Aerial conidia disperse and land on diverse substrata (1). If the conditions are suitable, the conidia will germinate (2) and the hyphae will proliferate (3). If there is not a suitable host, once the nutrients are used up, the hyphae will generate conidiogenous cells, produce aerial conidia and the cycle will repeat (7). If there is a suitable host, the hyphae will proliferate over the surface of the cuticle (3) until a suitable entry point is found. The hyphae will then digest their way through the layers of the cuticle (4) and enter the hemocoel. Within the hemocoel, growth switches from hyphal to yeast-like (5), and yeast-like hyphal bodies (blastospores) proliferate throughout the host. When the host dies, the switch occurs again and the resulting hyphae grow out through the insect cuticle (6), upon emerging from the host the hyphae produce conidiogenous cells, and the cycle repeats (7).
Biological Control

Fungal Biocontrol Agents

Fungi are important in limiting insect populations in nature and over 700 entomopathogenic fungal species, predominantly hyphomycetes have been described. The effective impact on host populations depends on host range and the ability to attack specific life stages. These attributes vary greatly by species and in some cases by strain (81). A number of commercial mycoinsecticidal preparations containing *Verticillium lecanii, Metarhizium anisopliae, Beauveria bassiana, B. brongniartii, M. flavoridiahave, and Paecilomyces fumosoroseus* have been developed and used with some success in Holland, Australia, Brazil, Germany, France, the former USSR, the former Czechoslovakia, Switzerland, Austria, Australia, and the UK (81). The targets of these formulations are diverse, with whiteflies, aphids, coffee berry and corn borers, grasshoppers, locusts, Colorado beetles and cockchafers as the most targeted organisms.

Some commercial mycoinsecticides have been developed in the US, however several factors have hampered widespread use including complex large-scale production problems and potential loss of viability if precise production and storage conditions are not met (89). Commercial mycoinsecticidal efficiency is sensitive to environmental conditions, especially low humidity levels. Even in soil applications, where exposure to ultraviolet radiation and desiccating conditions are limited, competition with soil microbes can limit the efficiency of fungal based insecticides (89).

*Beauveria bassiana as a Biocontrol Agent*

Commercial insecticidal formulations based on *B. bassiana* have been developed targeting diverse organisms including beetle larvae, plant hoppers on rice, grasshoppers, whiteflies, and locusts. As with other commercial mycoinsecticides, despite the
advantages, including wide host ranges, low environmental persistence (desirable in application practices), mass production, and a wider range of target hosts not effectively targeted by other biocontrol agents (viruses, bacteria, nematodes etc.), there are still a number of obstacles to widespread applications.

The highest success of commercial mycopesticides, including \textit{B. bassiana} based products, is in third world countries where lower production costs and fewer governmental regulations make the products more cost effective (37). In the US Companies such as Mycotech and Troy biosciences have developed and are marketing Mycotrol® and Naturalis® which are \textit{B. bassiana} based formulations.

Information relating to how different \textit{beauveria} cell types initiate the infectious processes, combined with the development of efficient methodologies to transform, genetically manipulate, and characterize filamentous fungi at a molecular level, will significantly affect the development of improved mycoinsecticides which in turn could lead to increased use of these products.

\textbf{Factors Affecting Adhesion}

Intermolecular forces between biological molecules involve complex interactions. In addition to the intermolecular effects of biological structures such as membranes, cell wall structures, or the entire organism acting in a non-localized, non-specific manner, interactions derived from the influence exerted by proteins, lipids, carbohydrates, and other molecules that can have localized receptor-ligand binding in addition to electrostatic and hydrophobic/hydrophilic interactions also need to be taken into account. The parameters that influence adhesion interact with each other in non-linear manners and can involve competing, synergistic or highly interdependent dynamic interactions.
Van der Waals forces, permanent dipole-dipole interactions, H-bonding, hydration forces, hydrophobic interactions and entropy-driven forces mediate van der Waals interactions. Some of these forces are short ranged and are primarily involved in adhesion and binding, whereas long-range forces are involved in colloidal aggregation. For descriptive purposes biological intermolecular reactions will be separated into two main categories, non-specific and specific binding, although some processes may involve both types of interactions intermolecular (59).

**Specific Binding**

Biospecific interactions are a subclass of highly complementary, non-covalent bonds. They are characterized by the close chemical and geometric fit between ligand and receptor binding pocket and entail relatively strong binding energies. These high bond energies \(10^{15} \text{ m}^{-1}\) for biotin/avidin) (95) are highly dependent on very small distances between the ligand and receptor.

Specific binding is dependent on distance and acts primarily when molecules are very close to each other. The molecules are brought into close proximity by the action of long-range forces, which guide the ligand into the specific receptor. Similar forces govern both specific and non-specific biological interactions. Specific forces tend to be attractive, relatively small and heterogeneous, whereas non-specific forces are usually repulsive, influence large areas, and the interacting surfaces tend to be more homogeneous intermolecular (59).

**Nonspecific Binding**

Non-specific interactions important for biological intermolecular forces include electrostatic interactions, steric interactions, and hydrophobic interactions. Electrostatic
interactions can be further subdivided into van der Waals interactions, permanent diapoles, and hydrogen bonding.

Electrostatic interactions are repulsive or attractive interactions that occur between ions and charged surfaces. Van der Waals, diapole-diapole, and hydrogen bonds are relatively weak attractive forces between temporary or permanent diapoles. At one point, the definition of van der Waals forces encompassed all intermolecular forces, but presently only London (dispersive) forces are included. London forces are transitory forces generated when electron clouds of different molecules oscillate in unison creating attractive forces between the molecules. These forces are transitory, aligned with each other and are dependent on the size of the molecule, the larger the molecule the stronger the force.

The diapoles in London forces are derived from non-polar molecules and are induced and temporary in contrast interactions between compounds with permanent diapoles (polar molecules) lead to increased adhesion and cohesion. This explains the observed higher boiling points for polar liquids, as compared to non-polar liquids. If hydrogen atoms are attached to small electronegative groups such as O, N, or F, the diapoles generated are bigger and the resulting interactions (H-bonds) are proportionally stronger.

**Steric, Bridging and Depletion forces**

Flexible polymer-like groups are present on the surfaces of many microbes; these include polysaccharides, tethered ligands, or lipids, which mediate many biological interactions. The interactions mediated by these groups can be repulsive if polymer-like groups are tethered to the surface, coiled or do not interact strongly with each other, the bulkier the groups, the stronger the repulsion. These polymers can initiate adhesion to
other surfaces via attractive forces if the ends and/or the whole length of the tethered
polymers are linked to functional groups, as the guest molecule approaches they can
initiate the binding process.

When polymers (such as polyethylene glycol, PEG) are free in solution they can
also mediate adhesion of biological surfaces by resulting in depletion forces arising from
the osmotic pressure between the bulk solution, and the zone between the two surfaces
devoid of polymer (depletion zone). Water is driven out of the depletion zone and into
the bulk solution thus forcing the two surfaces together. This is the principle behind
using PEG in cell fusions and transformations (59).

**Hydrophobic Interactions**

Hydrophobic interactions are considered to be the most important interactions of
molecules dissolved in highly polar liquids (87). It is considered the predominant
interaction mediating microbial binding to biosurfaces and Boucias et al. (1998) noted
that it probably was the predominant interaction mediating fungal adhesion to insect
cuticle. The actual mechanisms behind hydrophobic interactions are still not fully
understood, however, if a non-polar liquid is dissolved in polar liquid droplets of the
former will rapidly aggregate. This gives the appearance that non-polar molecules ‘fear
water, hence the name water fearing (hydrophobic) substances.

Hydrophobic interactions can be best described as the exclusion of hydrophobic
molecules from water resulting in the squeezing together effect of these surfaces.
Hydrophobic forces are relatively strong and long ranged, more so than other non-
specific intermolecular forces by a factor of 5-10 times (59).

The driving force behind hydrophobic interactions is the high free energy of
cohesion of water. According to Van Oss, Lifshitz-van der Waals (LW) forces have little
or no effect on hydrophobic interactions, because hydrophobic materials have apolar (LW) surface tension components \( (\gamma_{wLW}) \) that are close to that of water \( (\gamma_{wLW}) \) of water @ 20 °C = 21.8 mJ m\(^{-2}\), the \( \gamma_{iLW} \) of completely apolar materials range from 18 to 33 mJ/m\(^{2}\).

This results in LW free energies of attraction \( (\Delta G_{iwiLW}) \) at close range between water and the material, of 0 to –2.3 mJ/m\(^{-2}\). The Lewis acid-base surface energy components \( (\Delta G_{iwiAB}) \) are much larger (up to –102 mJ/m\(^{-2}\)). Because the total interfacial energy is equal to the combined values of the LW and AB components, the contribution from LW tends to be very low (87).

Hydrophobic compounds do have an affinity for water with \( \Delta G_{iw} \) values of –40 to –55 mJ/m\(^{2}\), but this affinity is not strong enough to overcome the high cohesive energy of water. Hydrophilic substances, on the other hand, have higher affinities for water \( (\Delta G_{iw}) \) values of –113 to –143 mJ/M\(^{2}\)). Hydrophilic interactions are characterized by a net repulsion between similar hydrophilic surfaces in water. This repulsion can occur only if the surfaces have a stronger adhesion to water than the polar free energy of cohesion of water \( (\Delta G_{w} = - 102\ \text{mJ/M}^{2}) \) (87).

Specific Molecules or Structures Involved in Fungal Adhesion

The forces regulating spore-cuticle initial interactions between entomopathogenic fungi and hosts are preexisting involving various combinations of specific and non-specific interactions. Specific interactions can involve: (1) the recognition of specific carbohydrate groups present on the insect cuticle by lectins (carbohydrate binding proteins), (2) protein-lipid binding, (3) protein-protein binding, and (4) other receptor-ligand binding. Non-specific interactions are mediated by hydrophobic and/or electrostatic interactions.
Freeze etched carbon replicas of the aerial conidial surfaces visualized with electron microscopy (EM) (52, 68, 96), and more recently Atomic Force Microscopy (AFM) (Kirkland & Keyhani, unpublished data), (27), have shown that hydrophobic aerial conidia from various fungi possess a well organized, uncoated, outer layer of hydrophobic rodlets. These rodlets are predominantly composed of hydrophobins small, secreted, hydrophobic proteins characterized by eight conserved cysteine residues and the ability to self-assemble into amphipathic membranes. These proteins are ubiquitous in ascomycete and basidiomycete fungi, but have not yet been found in other phylogenetic groups. They are involved in a variety of functions including spore coat protection, escape of aerial structures from water, virulence, and signaling (90).

Most other fungal cell types, including hydrophilic conidiospores, either lack hydrophobin layers, coat the hydrophobin layer with mucilage (obscuring visualization), or possess very disorganized, uncoated rodlet layers (increasing organization, being correlated with increasing hydrophobicity) (96).

For some hydrophilic fungi, preexisting mucilaginous coats or mucus released at the time of cuticle contact mediate attachment to hosts. Fungi that produce motile hydrophilic spores promote attachment by secreting adhesion vesicles from pseudopodia when in contact with the insect cuticle; others release mucilage from germ tubes, or appressoria, in order to consolidate adhesion (91).

Understanding the mechanisms that underlie aerial conidial adhesion to insect cuticles and other surfaces is important, because this is the initial and thus a crucial step in pathogenesis. Aside from aerial conidia, B. bassiana produces at least two other single cell propagules that have the potential to initiate infection. Knowledge concerning the
biochemical and biophysical mechanisms that mediate the adhesive properties of aerial conidia and other specialized cells can be a basis for making rational decisions on cell formulations optimized towards specific targets. This information is important in understanding the biology and ecology of *B. bassiana*, and can have an impact on practical considerations for the use of *B. bassiana* as a biological control agent.

**Molecular Biology**

**Methods Used to Study Global Differential Gene Expression in Fungi**

Increasingly the information about the surface properties of fungi, including factors affecting pathogenicity and virulence, which could lead to technologies that may improve the efficacy of mycoinsecticides, will be found by analyzing the organisms at a molecular level. Understanding which genes are important for the infection process may lead to the development of commercial insecticidal products specifically designed to adhere better, with higher infection rates and/or virulence, this in turn will decrease the infective doses leading to more cost effective products.

Genes important for the infectious process are carefully regulated (either up or down) when the parasite comes into contact with the host. Comparison of the concentrations of individual mRNAs in samples obtained from cells, which have been exposed to different conditions, or which have been genetically altered, can yield valuable information about the spatial and temporal relationships of gene expression. Isolation of differentially expressed transcripts can lead to the characterization of regulatory networks and motifs (promoters and other cis elements). Comparative analysis of transcript and proteome data can provide further information about how protein levels are regulated.
There are a number of methods used to analyze differential expression of multiple genes (transcriptome analysis). These techniques include array technology, serial analysis of gene expression (SAGE), techniques based on amplified fragment length polymorphism (AFLP), differential display, reverse transcription PCR (DDRT-PCR), differential screening of cDNA libraries, and reverse transcriptase, real time PCR (reverse transcriptase RT-PCR). Some of these methods are combined to improve upon the limitations of the individual techniques.

**Differential screening of cDNA libraries**

Screening cDNA libraries is the classical method of isolating differentially expressed cDNAs. Successful application of this method is limited unless the mRNA of interest comprises at least 0.05% of the total RNA in one cell line, and less than 0.01% in the other (55). Subtractive hybridization (SH) is specifically designed to remove common expressed sequences, increasing the concentration of differentially expressed sequences in the probe, and therefore the overall specificity of the procedure. SH technology involves hybridizing cDNA from treated sample libraries (tester cDNA) to cDNA from control sample libraries (driver cDNA). The DS, hybridized cDNA is removed, leaving behind SS molecules representing genes that are expressed at a higher level in the tester population.

A further improvement to this technology is Subtraction Suppressive Hybridization (SSH); a PCR based technique that normalizes, subtracts, and amplifies message expressed at higher levels in the tester cDNA library without the requirement of physically separating DS cDNA from SS cDNA. Unlike SH, SSH does not require multiple rounds of hybridization and the normalization and subtraction steps are combined in a single reaction (23, 36, 61, 64).
cDNA from the experimental groups (tester), and from the subtracting cDNA (driver) are digested with a four base cutter. Then tester cDNA is split equally into two aliquots, which are then ligated to different adaptors. Each tester sample is mixed with excess driver, denatured and allowed to anneal. Normalization (equalization) of sequence abundance occurs during this step because high abundance sequences will self-anneal faster than low abundances reducing the percent of high abundance differentially expressed molecules in the SS cDNA pool. The tester populations are mixed together in the presence of excess driver, this further enriches for differentially expressed SS cDNA, but it also allows the formation of hybrid molecules with different adapters at each end (heterohybrids). The ends of the molecules are then filled in to generate primer-annealing sites.

During the PCR amplification only these heterohybrids (tester1/tester2) are exponentially amplified, whereas homohybrids (tester1/tester1, tester2/tester2 and driver/driver) will not be amplified. The tester homoybrids have identical adapters with long inverted terminal repeats that form self anneal at temperatures higher than the annealing temperatures of the primers and forming panhandle structures which cannot be amplified (Fig. 1-2).

![Adapters](image)

**Adapters**

**cDNA molecule**

Fig 1-2. Panhandle structure due to annealing of identical adapters

Driver/driver homohybrids have no adapter and cannot be amplified. Driver/tester hybrids will be amplified linearly, and none of the single stranded molecules will be amplified because they don’t have adapters, or they don’t have primer-annealing sites.
The end result is amplified sequence fragments representing differentially expressed sequences, normalized with respect to sequence abundance in the original sample. These fragments can then be cloned into appropriate vectors for further analysis. This technique was used to identify *B. bassiana* genes up regulated during the initial stages of pathogenesis; it was hypothesized that if hydrophobins were important in the infection process they might be upregulated at this stage.

**Real Time RT-PCR**

The polymerase chain reaction (PCR) has three basic reaction rates. Initially, when reagents are not limiting, the reaction proceeds exponentially; eventually one or more reagent becomes limiting and the reaction proceeds in a linear manner; and finally very little new product is made and amplification rates reach a plateau.

Using PCR to accurately quantify DNA relies on collecting the data during the exponential stage and having a large enough dynamic range (several orders of magnitude). Real time PCR automates this procedure by automatically generating and plotting data points from all samples during each amplification cycle, yielding a dynamic range of about $10^7$ fold. Data analysis, standard curve generation, and copy number calculations are also automated. Converting mRNA to cDNA prior to PCR amplification and amplifying specific genes within samples yields information about differential abundance of mRNA species under different conditions.

Real time RT-PCR products are detected fluorescently. There are currently four basic technologies used for this purpose: Taqman® (Applied Biosystems), Molecular beacons, Scorpions®, and SYBR® Green (Molecular probes). All these technologies, except SYBR green, rely on Förster Resonance Energy Transfer (FRET) to quench fluorescently labeled probes until they have hybridized with the template. This quenching
is accomplished by physically restraining the fluorophore in close proximity to the quencher. Once the probe is amplified, the quencher and probe are separated by a variety of processes including release of the fluorophor, and inhibition of hairpin loops present in free unhybridized probes. Because fluorescence is dependent on template-probe hybridization increases in the quantity of template results in equivalent increases in fluorescence from cycle to cycle.

SYBR® green is a fluorescent dye which fluoresces strongly in the presence of double stranded DNA. As with the other techniques, the level of fluorescence increases proportionally to the amount of PCR product. SYBR-green based technologies tend to be less expensive than probe based systems, but more sensitive to errors derived from the amplification of non-specific products or the presence of primer dimers. Although the costs associated with fluorescent probes tend to be higher, there is the added advantage of being able to perform multiplex PCR; where DNA species within the same sample are amplified and differentiated by labeling the probes with differently colored fluorescent molecules.

Once differential abundance of message for specific genes is observed and confirmed, the importance of these genes in the processes of interest needs to be verified. This often requires targeted gene manipulation (knockout) of wild-type genes and characterization of the resultant mutants.

**Objectives**

The objectives of this research include: (1) quantification of the relative contributions of surface forces in the adhesion processes of *Beauveria bassiana* single cell propagules to solid surfaces; (2) analysis of the hydrophobic and electrostatic cell surface properties of *B. bassiana* single cell propagules; (3) the development of a
quantitative assay to measure microbial adhesion to surfaces; (4) molecular characterization of proteins involved in *B. bassiana* adhesion to solid surfaces; and (5) analysis of the mRNA levels of these molecules in different cell types and under varying growth culture conditions.
CHAPTER 2
ADHESION OF THE ENTOMOPATHOGENIC FUNGUS BEAUVERIA BASSIANA TO SUBSTRATA

Introduction

Under intensive study for use as a biopesticide, the entomopathogenic fungus *Beauveria bassiana* displays a broad host range able to target a diverse number of arthropod species. Strains of *B. bassiana* have been selected for control of insects and other arthropods that act as disease vectors including mosquitoes and ticks (14, 54); crops pests such as whiteflies, caterpillars, grasshoppers, and borers (12, 20, 53, 58, 100); and even ecologically hazardous, invading pests such as fire ants and termites (11, 17). The varied cuticles of these organisms represent the first barrier to the pathogen, and attachment of fungal propagules to the cuticle is the initial event in establishing mycosis. Air currents, dispersion via water droplets, as well as saprophytic growth over substrata inhabited by insects are considered the major routes for contact of fungal spores with host cuticles. Upon contact, fungal cells bind to the cuticle and initiate a developmental program that includes the production of specialized infection structures such as germ tubes and penetrant hyphae (9, 42). If the infection is successful, the fungus will grow across the cuticle surface, penetrating the host cuticle to invade and proliferate within the hemolymph, ultimately resulting in the death of the host.

Fungal cell attachment to cuticle may involve specific receptor-ligand and/or non-specific hydrophobic and electrostatic mechanisms (9, 10, 25). A haploid anamorphic fungus, *B. bassiana*, produces a number of mono-nucleated single cell types including
aerial conidia, blastospores, and submerged conidia that can be isolated from agar plates, rich broth submerged cultures, and nutrient limited submerged cultures, respectively. Although it is well known that culture conditions (and hence the cell type produced) can affect successful virulence towards targeted hosts, little is known about the adhesion process of *B. bassiana* cell types other than conidia. This report describes a quantitative assay used to determine the binding qualities and adhesion substrata preferences of *B. bassiana* aerial conidia, blastospores, and submerged conidia.

**Materials and Methods**

**Cultivation of Fungi**

*Bauveria bassiana* (ATCC 90517) was routinely grown on potato dextrose agar (PDA) (4). Plates were incubated at 26°C for 10-14 days and aerial conidia were harvested by flooding the plate with sterile dH₂O. Conidial suspensions were filtered through a single layer of Mira-cloth (Clabiochem, CA) and final spore concentrations were determined by direct count using a hemocytometer. Blastospores were produced in Difco™ Sabouraud dextrose (Becton, Dickinson and Co., MD)+ 1-2% yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of 0.5-5 x 10⁵ conidia/ml as the inoculum. Cultures were grown for 3-4 days at 26°C with aeration (150-200 rpm). Cultures were filtered (2x) through glass wool to remove mycelia, and the concentration of blastospores determined by direct count. Submerged conidia were produced in TKI broth using fructose as the carbon source as described by Thomas et al. (1987). For all cell types, Mira-cloth (Clabiochem, CA) or glass wool filtered cell suspensions were harvested by centrifugation (10,000xg, 15 min, 4°C), washed two times with sterile dH₂O, and resuspended to the desired concentration as
indicated (typically $10^7$-$10^8$ cells/ml) in water for final dilution as required for the experiment.

**FITC-labeling of B. bassiana Cells**

Fluorescein isothiocyanate (100 µl of 1mg/ml stock solution per ml of fungal cells) was added to washed fungal cell-types (0.5-1x$10^8$ cells/ml) resuspended in 50 mM calcium-carbonate buffer, pH 9.2. The reaction was incubated for 20 min in the dark, after which the cells were extensively washed (4-5 times with volumes equal to the original volume) with TB (50 mM Tris-HCl, pH 8). Final cell pellets were resuspended in TB (pH 8) to the desired concentrations as indicated. Final single cell propagule concentrations were checked by direct count using a hemocytometer.

**Adhesion Assay**

Two assays were used to assess adhesion to substrata. In the first (qualitative), fungal cell suspensions (100 µl, 1-20 x $10^6$ cells/ml) were spotted and incubated in slide chambers (treated and untreated glass surfaces, Lab Tech chamber slide system, Nalgene Nunc, Naperville, Il), at 25°C and 100% humidity for various periods of time. Adhesion was assessed microscopically after a 1x wash or 3x wash in TB (pH 8). Digital images were taken using a Nikon Optiphot-2 microscope with a digital camera. Adhesion was also assessed quantitatively using FITC-labeled cells incubated in various black microtiter plate test substrata. Fungal cell suspensions (100 µl, 1-20 x $10^6$ cells/ml) were placed in (black) microtiter plate wells and incubated at 25°C in the dark for various periods of time. Unbound cells were removed by aspiration of the liquid from the wells followed by up to 3 washes with 450 µl TB (pH 8). Fluorescence was measured using a Spectra Max Gemini XS microplate fluorometer (Molecular Devices Corp., Sunnydale,
CA), Ex wavelength: 495 nm, Em: 530 nm, cutoff: 515 nm. For each experiment a standard curve of fluorescent intensity versus cell number (as measured by direct count) was prepared. Typically, the fluorescent intensity was measured before wash (total number of cells) and after each wash. Weakly polar microtiter plates (Fluorotrac F200) and hydrophilic microtiter plates (Fluorotrac F600) from Greiner Biotech (Longwood, Fl) and were used unmodified. Hydrophobic substrata were prepared by addition of a thin layer of silicone using Sigmacote (Sigma Corp., St. Louis, Missouri) onto glass slides or into Fluorotrac F200 microtiter plate wells. Typically, substrata were treated up to three times with Sigmacote and the treated plates or slides were placed in a fume hood overnight to ensure solvent evaporation.

**Enzyme Treatments**

Aerial conidia, blastospores, and submerged conidia (0.5 – 1.0 x 10^7 cells/ml) were washed twice and resuspended in the manufacturer’s suggested enzyme reaction buffers. Stock solutions (100 µl of 10 mg/ml) of amylase (Sigma, A6255), cellulase (Sigma, C9422), or laminarinase (Sigma, L5272) in 0.01 M KPO₄, pH 6.8 for α-amylase and pH 5.6 for cellulase and laminarinase, were added to 0.9 ml cells resuspended in the same buffer. For protease treatments, 100 µl of a 10 mg/ml stock solution and 50 µl of a 1 mg/ml stock solution of Proteinase K (Sigma, p6911) and Pronase E (Stratagene, 300140) respectively, in buffer (0.1 M Tris HCl, pH 7.8, 0.5% sodium dodecyl sulfate (SDS), and 1 mM CaCl₂) were added to cells resuspended in the same buffer. Glycosidase reaction mixtures were incubated for 4 hr at 25⁰ C, and protease treatments were performed for 4 hr at 37⁰ C. After incubation, cells were extensively washed in 50 mM calcium bicarbonate buffer pH 9.2 (7-8 times, 1 ml each) by centrifugation (10,000 x g, 5 min).
Treated, washed cells were then FITC-labeled and used in adhesion assays as described above.

**Effect of pH Influence on Attachment**

FITC-labeled cell types (0.5 – 1.0 x 10^7 cell/ml) were washed twice and resuspended in one of the following physiological buffers (0.1 M): acetate (pH 4 and 5), MES (pH 6 and 7), HEPES (pH 7 and 8) and TB (pH 8) before being used in adhesion assays. Control wells with cells suspended in TB (pH 8) were used to determine initial cell concentrations due to the pH sensitivity of fluorescence intensity measurements. Normalization due to pH effects on the FITC intensity was achieved by allowing adhered cells (i.e. after the adhesion assay incubation and washing steps) to equilibrate in TB buffer (pH 8) until the fluorescence intensity of signal of the cells stopped increasing.

**Competition Assays**

Cells were FITC labeled, and the final cell pellets resulting from the washing steps of the labeling reaction were suspended in TB containing 0.3 M carbohydrate (added as a competitor), 0.1% detergent (SDS, Tween 80 or CTAB), or 1 M NaCl solution. Cells were immediately used in adhesion assays.

**Contact Angle Determination**

Contact angle measurements of the surfaces used to evaluate the adhesive properties of the fungal cell types were determined using a Ramé-hart Model 500 Advanced Goniometer with automated drop dispenser and tilting plate running DropImage Advanced software (Ramé-hart). Dynamic angle measurements were determined just prior to movement of the water drop. Briefly, a 10 µl drop of sterile water was placed onto the surface of the substrata to be tested. The stage and the camera were tilted at 10°C increments until the drop was on the verge of movement. The leading
edge (dynamic) contact angle was determined from the last measurement taken prior to drop movement.

Results

A quantitative assay was developed to measure the kinetics of fungal cell adhesion to various substrata. Fungal cells chemically treated with the fluorescent reagent FITC, appeared uniformly labeled, with clear halo rings defining the cell wall (Fig. 2-1). Labeling of all three cell types, aerial conidia, blastospores, and submerged conidia displayed a linear relationship between cell number (as measured by cell counting using a hemocytometer) and fluorescence intensity (Fig. 2-2). Little variation was observed within experiments (each point in Fig. 2-2 represents triplicate samples); however, some variation (up to 2-fold in fluorescence intensity) was observed between separate FITC labeling reactions for each experiment. Therefore, a standard curve of fluorescence intensity versus cell number, as determined by cell counting, was determined and used for each experiment.

Fig. 2-1. Bright field (A, B, C) and fluorescent microscopy (D, E, F) of FITC-labeled *B. bassiana* aerial conidia (A, D), blastospores (B, E) and submerged conidia (C, F). Bar = 5 µm (A) and (B) and 10 µm (C).
In order to determine the effects of the labeling reaction on the adhesive qualities of the cells, a series of preliminary qualitative experiments was performed using untreated and silinized glass slides with both unlabeled (not shown) and labeled cells (Fig. 2-1). In all instances no difference was observed using FITC-labeled or unlabeled cells (data not shown). These experiments demonstrated: (1) that aerial conidia were able to bind to hydrophobic, but not hydrophilic surfaces (Fig. 2-3), (2) blastospores bound uniformly to hydrophilic surfaces, but bound poorly to hydrophobic surfaces, forming small clumps on the latter (Fig. 2-4), and (3) submerged conidia bound equally well to both hydrophilic and hydrophobic surfaces, forming large clumps that appeared more evenly distributed over time (Fig. 2-5). These patterns were identical between FITC-labeled and unlabeled cells (data not shown).

For the quantitative assays, three types of black polystyrene based microtitre plates with differing surface characteristics were used as substrata: (1) siliconized Fluorotrac-F200, highly hydrophobic, (2) F200 untreated polystyrene surface, weakly polar, and (3) F600, treated polystyrene, hydrophilic, polar surface containing hydroxyl, carbonyl, and amino groups with a small net negative charge. Dynamic leading edge water droplet contact angle ($\theta$) measurements of the three substrata agreed with their decreasing hydrophobicity. The silinated F-200 plates displayed a $\theta = 104.7^\circ$, the untreated F-200 plates a $\theta = 95.6^\circ$, and the F-600 plates a $\theta = 85.6^\circ$ (contact angles for cleaned polished glass, the glass chamber slides, and silinated glass were determined to be: 73.1, 87.4, and 109.7, respectively).
Fig. 2-2. Fluorescent intensity of FITC labeled aerial conidia ($\zeta$), blastospores ($\mu$), and submerged conidia ($\lambda$) as a function of cell number.

Fig. 2-3. Adhesion of aerial conidia to glass (A1, A2, A3) and silinated glass (B1, B2, B3) surfaces after 5 min (A1, B1), 4 hr (A2, B2), and 24 hr (A3, B3) incubation on substrata. Bar = 30 µm.
Fig. 2-4. Adhesion of blastospores to glass (A1, A2, A3) and silinated glass (B1, B2, B3) surfaces after 5 min (A1, B1), 4 hr (A2, B2), and 24 hr (A3, B3) incubation on substrata. Bar = 30 µm.

Fig. 2-5. Adhesion of submerged conidia to glass (A1, A2, A3) and silinated glass (B1, B2, B3) surfaces after 5 min (A1, B1), 4 hr (A2, B2), and 24 hr (A3, B3) incubation on substrata. Bar = 30 µm.

The number of binding sites per microtiter plate well was estimated at 4-8 x 10^5 cells as determined by the saturation point derived from plots of the percent cell bound as a function of cell concentration (Fig. 2-6). These data indicated that the linear range of
each cell type was similar, although the saturation point for each cell type varied from approximately 25% of the aerial conidial cells able to bind per well, to greater than 70% of the submerged conidia bound in wells using ~5 x 10^5 cells/well. All subsequent experiments were performed using cell concentrations within the linear range of the attachment curve (2-5 x 10^5 cells/well) (Fig. 2-6).

Quantitative adhesion assays were performed using aerial conidia, blastospores, and submerged conidia on hydrophobic, weakly polar, and hydrophilic surfaces with either 1 or 3 washes (Fig. 2-7). Aerial conidia bound rapidly and tightly to hydrophobic surfaces, with no loss of cell binding by up to 10 washes with buffer (data not shown). Aerial conidia bound poorly to weakly polar surfaces even after prolonged (24 hr) exposure to the substrata. Interestingly, these cells bound weakly to hydrophilic surfaces and were readily washed off indicating that this binding process might be biphasic, with initial weak electrostatic binding.

![Figure 2-6](image-url)

Fig. 2-6. Saturation point of binding sites for *B. bassiana* aerial conidia (○), blastospores (□), and submerged conidia (O) to microtiter plates
In contrast, blastospores bound poorly to hydrophobic surfaces with no greater than 10% of the cells bound even after 24 hr. Blastospores bound moderately to weakly polar surfaces, with approximately 1-2 x 10^5 cells bound/well (30% of 5 x 10^5 cells used in the assay) after 4 hr incubation. Blastospores bound more readily to hydrophilic surfaces, with up to 50% of the cells bound within 30 min. Submerged conidia displayed the broadest binding characteristics, adhering to all three surfaces, although with slightly differing kinetics. On hydrophobic, weakly polar, as well as hydrophilic surfaces up to 60% of the cells used bound to the substrata within 4 hr, although in the case of the hydrophobic and weakly polar surfaces, almost half of the bound cells could be removed using three washes.

Fig. 2-7. Quantitative adhesion and influence of washing on adhesion of aerial conidia, blastospores, and submerged conidia to silinated F-200 (hydrophobic), F-200 (weakly polar), and F-600 (hydrophilic) microtiter plates. Dark violet bars represent a single wash; maroon bars represent a triple wash.
Attachment of aerial conidia to hydrophobic surfaces could not be competed with any of the carbohydrate compounds tested including glucose, galactose, lactose, maltose, melibiose, or trehalose and was insensitive to salt concentrations as high as 1 M NaCl (Table 2-1). The presence of N-acetylglucosamine appeared to promote adhesion (confirmed by microscopic analysis). The affect was not due to any visible growth or mucilage production, i.e. no germination/germ tube or secretion of extracellular matrix was observed. Conidial attachment was, however sensitive to the presence of detergents, inhibition (80-90%) could be observed using 0.1% Tween-80 (non-ionic detergent), CTAB (cationic detergent), or Triton-X 100 (non-ionic detergent).

Table 2-1. Effects of various competitors and chemicals on *B. bassiana* cell-type adhesion

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aerial conidia</th>
<th>Attachment Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Blastospires&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Submerged conidia&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>2.0 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.0 ± 0.2</td>
<td>1 ± 0.2</td>
<td>1 ± 0.1</td>
<td>nd&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Melibiose</td>
<td>1.0 ± 0.2</td>
<td>1 ± 0.2</td>
<td>nd&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>Maltose</td>
<td>1.0 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>1 ± 0.2</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1 ± 0.1</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1 ± 0.2</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>0.2% Tween-20</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>0.2% SDS</td>
<td>0.4 ± 0.5</td>
<td>0.8 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0.2% CTAB</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0.2% Triton-X 100</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Attachment ratio = (% cells bound under condition tested)/(% cells bound under control conditions)
<sup>b</sup> Cells tested on siliconized F200 (hydrophobic) microtiter plates.
<sup>c</sup> Cells tested on F600 (hydrophilic) microtiter plates.
<sup>d</sup> nd, not determined

The anionic detergent SDS also inhibited conidial attachment to hydrophobic surfaces although a large degree of variation was observed. Adhesion of aerial conidia to surfaces was only slightly affected by pH with 30% less cells bound at pH 4.0 than at pH 7.0 (Table 2-2).
A unique feature of blastospore attachment was that adhesion could be competed with maltose (Table 2-1). No other sugar tested had any affect on blastospore adhesion nor did maltose affect conidial or submerged conidial adhesion. Blastospore attachment was insensitive to salt (NaCl), SDS and CTAB, but was inhibited by Tween-20 and Triton X-100. In contrast to the other cell types, adhesion of blastospores appeared to be pH dependent with a 50% decrease in the number of cells bound when assays were performed at pH values of 4-5 compared to pH 7-8 (Table 2-2). Submerged conidia behaved similar to aerial conidia except that N-acetylglucosamine did not increase the number of cells bound and the presence of the detergents CTAB and Triton X-100 (Table 1) and changes in pH had only a minor affect on submerged conidial adhesion.

Table 2-2. Effect of pH on *B. bassiana* cell-type adhesion

<table>
<thead>
<tr>
<th>pH</th>
<th>Aerial conidia</th>
<th>Blastospores</th>
<th>Submerged conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.4</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

a) Attachment ratio = (% cells bound under condition tested)/(% cells bound under control conditions)
b) Cells tested on siliconized F200 (hydrophobic) microtiter plates.
c) Cells tested on F600 (hydrophilic) microtiter plates.
d) nd, not determined

Removal of carbohydrates (maltose, glucose or glucuronic acid) from the cell surface of aerial conidia using either α-amylase or laminarinase, but not cellulase resulted in decreased conidial adhesion to hydrophobic surfaces but had no affect on conidial adhesion to hydrophilic surfaces (Table 2-3). Treatment of blastospores with glycosidases appeared to either slightly promote adhesion (α-amylase and to a lesser extent cellulase treatment) to hydrophilic surfaces or not affect adhesion (laminarinase
and/or hydrophobic surface). Glycosidase treatment of submerged conidia resulted in a 25-50% decrease in adhesion to hydrophilic surfaces and hydrophobic surfaces. The only other remarkable difference was noted in the adhesion of α-amylase, treated cells to hydrophobic surfaces where a large variation in adhesion was noted.

Some differential effects were observed by protease treatment of the cell types (Table 2-3). Aerial conidia treated with Pronase E displayed a greater than 50% loss of adhesion to hydrophobic surfaces but no loss in adhesion to hydrophilic surfaces, although a large variation was observed. This variation was between experiments, i.e. different cell batches treated with the enzyme and may reflect surface heterogeneity or accessibility of target substrates to the enzyme. Protease K treatment of aerial conidia did not result in appreciable changes in adhesion. Similar treatment of blastspores with proteases had no effect or resulted in an almost 2-fold apparent increase in adhesion. Microscopic analysis (i.e. visual counting) of the number of cells bound indicated that there did not appear to be an actual increase in the number of cells bound and instead protease treatment appeared to increase the fluorescence intensity signal.

Table 2-3. Effects of various enzymatic treatments on B. bassiana cell-type adhesion

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Aerial conidia hydrophobic</th>
<th>Aerial conidia hydrophilic</th>
<th>Blastospores hydrophilic</th>
<th>Submerged conidia hydrophobic</th>
<th>Submerged conidia hydrophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-amylase</td>
<td>0.3 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.15</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Cellulase</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.7</td>
<td>1.3 ± 0.05</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Lamminarinase</td>
<td>0.3 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Pronase E</td>
<td>0.4 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>nd</td>
<td>nd</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Protease K</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

a) Attachment ratio = (% cells bound under condition tested)/(% cells bound under control conditions)
b) Cells tested on siliconized F200 (hydrophobic) microtiter plates.
c) Cells tested on F600 (hydrophilic) microtiter plates.
d) Apparent increase (see text for details).
These were the only conditions tested in which the fluorescent signal was affected by the treatment. In all other experiments, microscopic analysis was in agreement with the fluorescent intensity measurements. Finally, little to no effect was observed for the adhesion properties of protease treated submerged conidia.

**Discussion**

Conidial adhesion has been examined in a number of plant and insect pathogenic fungi (9, 65). Adhesion of entomopathogenic fungi appears to involve an initial binding interaction followed by a consolidation step, resulting in firm attachment to the cuticle (9, 33, 34). Similarly, studies on the phytopathogenic fungus *Botrytis cinerea* revealed a two-stage adhesion process; immediate adhesion occurring upon hydration and characterized by relatively weak attachment and a stronger delayed adhesion observed as the conidia germinate (25, 26). Immediate adhesion of *B. cinerea* is passive (nonmetabolic) and although no specific structures were visible on the conidia, adhesion is characterized as dependent (in part) on hydrophobic interactions. Hydrophobic interactions have also been implicated in the attachment of conidia of the insect-pathogenic fungi *Nomuraea rileyi*, *Metarhizium anisopliae*, and *B. bassiana* to both host and non-host cuticle preparations (9).

*B. bassiana* produces at least three single cell types that can be distinguished based upon morphological and adhesive characteristics. Experiments qualitatively assessing entomopathogenic fungal adhesion to various surfaces including insect cuticles (9, 10, 25, 26, 42) have almost exclusively addressed conidial binding to surfaces and have not examined the adhesion properties of either blastospores or submerged conidia. Using a quantitative adhesion assay our results demonstrate complex interactions between various cell types and substrata with different surface properties. All three *B. bassiana* single cell
types studied (aerial conidia, blastospores, and submerged conidia) displayed different adhesion properties that appeared to be mediated by different cell-specific mechanisms.

*B. bassiana* aerial conidia were able to bind both hydrophobic and hydrophilic surfaces although adhesion to the latter was weak and the cells could readily be washed off. Aerial conidia binding to hydrophobic surfaces could not be competed with any carbohydrate tested, although addition of N-acetylglucosamine, the monomeric constituent of chitin (the major carbohydrate polymer found in arthropod cuticles) appeared to increase adhesion. The hydrophobic nature of *B. bassiana* conidial spores, as well as those from other entomopathogens such as *N. rileyi, M. anisopliae*, and *Paecilomyces fumosoroseus*, was correlated with the presence of an outer cell layer comprised of rodlets or fascicles as visualized by electron microscopy boucias (9, 10).

These rodlet layers are presumably formed by assembly of specific proteins termed hydrophobins, which in turn are thought to passively mediate adhesion to hydrophobic surfaces (92, 96). Although our results are consistent with this model, they also indicate that the interaction of aerial conidia with hydrophobic surfaces may be more complex. Since amylase and laminarinase as well as protease treatments reduced adhesion (but displayed no discernable effects on the rodlet layer, Kirkland, Holder, & Keyhani, unpublished results), both carbohydrates on the cell surface as well as proteins may be involved in mediating adhesion of aerial conidia.

In contrast, blastospores, cells lacking a visible rodlet layer (9, 10), bound poorly to hydrophobic surfaces forming small aggregates or clumps, but displayed high binding to hydrophilic substrata. Blastospores were also able to bind to weakly polar substrata, although a greater incubation time compared to hydrophilic substrata was required.
Intriguingly, blastospore attachment could be specifically competed with maltose. No other carbohydrate tested appeared to compete with adhesion of blastospores or the other fungal cell types including trehalose the major carbohydrate constituent found in insect hemolymph. *In-vivo* generated blastospores (distinct but similar to the rich broth produced blastospores), produced during fungal proliferation in the insect hemolymph after penetration of the cuticle, are able to evade recognition by insect hemocytes and display altered membrane characteristics (48, 49, 70). However, the physiological significance of potential maltose inhibition of adhesion of these cells is unclear.

Submerged conidia displayed the broadest binding characteristics of the *B. bassiana* single cell types, capable of efficiently binding hydrophobic, weakly polar, as well as hydrophilic surfaces. Spore tip or mucilage-covered appendages and adhesive knobs have been implicated to mediate conidial adhesion of several fungi (8, 45, 85). The mucosal coat of nematophagous fungi not only appears to mediate adhesion but is attractive to host insects. A wide variety of arthropod mycopathogens appear to produce exocellular mucilage during germ tube or appressorial formation (9). Similarly, the hydrophilic nature of conidia of the *Entomophthorales* is thought to be mediated by a mucilaginous coat released upon attachment to cuticle surfaces and that acts as a glue mediating attachment (30, 57). Although blastospores and submerged conidia attached to hydrophilic surfaces, no obvious mucilaginous coat was visible in either cell type. In addition, scanning electron microscopy did not reveal any specific structures in conidia, blastospores, or submerged conidia of *B. bassiana* that are predicted to be involved in mediating adhesion (unpublished data). It is possible, however that extracellular matrix
components or mucilage located between the inner and outer wall that is not readily detectable could be involved in mediating adhesion.

Although aerial conidia are considered easily dispersible via air currents, and are more resistant to adverse environmental conditions such as desiccation and extreme temperatures, submerged conidiation and blastospore formation may occur under a variety of environmental conditions as well as during the host-pathogen interaction. The ability to produce multiple cell types, with differing adhesive properties, may occur in response to specific environmental conditions.

This may allow fungal cells to bind to a broad range of host targets and provide the fungus with a means to adapt to substrata conditions (33, 34). It is unlikely, however that alteration in adhesion can account for emergence of restricted host-range *B. bassiana* strains, since these strains may have altered (cuticle degrading) enzyme production or may be unable to penetrate and/or respond to surface cues of certain hosts, but may still retain the means to initiate binding or adhesion interactions. Indeed, there is some evidence that when entomopathogens specialize they loose structures rather than gain them, It would be interesting to see whether there is any alteration in the adhesion kinetics of general and specialized strains of *B. bassiana*.

Our data indicates that certain practical considerations should be taken into account during application of *B. bassiana*. For instance, if blastospores were to be used, formulations should probably avoid hydrophobic solutions. Instead aqueous or polar liquids are recommended. Such formulations may prove to be more successful in the biocontrol of certain hosts as compared to aerial conidia.
CHAPTER 3
SURFACE CHARACTERISTICS OF THE ENTOMOPATHOGEN BEAUVERIA BASSIANA

Introduction

The entomopathogenic fungus \textit{B. bassiana} has a broad host range. The varied cuticles of these arthropod hosts are the first barriers to successful infection. Attachment of fungal propagules to the cuticle is the crucial initial event in establishing mycoses. \textit{B. bassiana} produces at least three distinct single celled propagules in response to differing growth conditions: Aerial conidia (AC), submerged conidia (SC) and blastospores (BS). These spores are distinct in morphology, genesis, germination characteristics, lectin adhesion profiles and tolerance of extreme environmental conditions (10, 72).

Adhesion factors typically found in other organisms, including glycoproteins and carbohydrates, are present on fungal spores or in the extra-cellular matrix surrounding most fungal structures. However, the non-specific physical properties important for adhesion are less well studied, especially for submerged spores (blastospores and submerged conidia). The net result of interactions between the surface of a solid and its surroundings is a function of the total surface free energy of the solid. According to Van Oss \textit{et al.} (1990), the total free energy (E) of a surface is the sum of three main components: acid/base interactions (AB), dispersive forces (LW) and electrostatic forces (EL) (Eq. 3-1)

\[ E_{\text{tot}} = E_{\text{LW}} + E_{\text{AB}} + E_{\text{EL}} \]  

(3-1)
Dispersive forces, acid/base interactions, and hydrophobic interactions can be calculated from contact angle data and by using Young’s equation, whereas the effective surface charge, or zeta potential, can be derived from the mobility (μ) of cells in an electric field.

Spores with rodlet layers or rough surfaces are more hydrophobic than smooth spores (39, 97). In our experiments atomic force microscopy (AFM) was used to generate high resolution images of the surfaces of the three spore types produced by B. bassiana. AFM is an example of proximity probe microscopy, where a cantilever, placed close the surface of the sample, is scanned in a raster (back and forth in an ordered manner) pattern to measure physical features such as height, elasticity, or hardness. A laser directed towards the cantilever is deflected, and the deflections are recorded on a positron-sensitive photodiode. As the probe follows the contours of the sample, it is attracted, or repulsed by it. The instrument measures the force of these interactions, and renders the output graphically. The resolution of the instrument, which depends on the size of the probe, ranges from 10 pm to 125 µm; the vertical limit of the sample is 5 µm.

In addition to high-resolution cell surface imaging, AFM can also generate data about surface biophysical properties. Two basic types of images are generated, height and deflection. The former gives accurate information about cell surface roughness and size, while the latter generates images with higher resolution for viewing ultra-structural details. In addition to these images, AFM can be used to generate force curves that can be used to measure local physical properties such as adhesion and elasticity.

AFM images can be correlated to electrostatic, or hydrophobic characteristics of the different spore types. The hydrophobic character of the spores can be determined
indirectly, by observing how well the spores attach to different surfaces, or directly, by quantitatively measuring hydrophobic indices. Past research has shown that different methodologies used to evaluate cell surface hydrophobicity do not always correlate well with each other, and can yield statistically significant differences for similar cells even from a single strain (2, 29, 46).

Phase exclusion assays, specifically microbial adhesion to hydrocarbons (MATH) and contact angles, have been used in a number of biological systems to obtain quantifiable hydrophobic indices. The advantages of this system include ease of use, reasonable reproducibility, extensive use in the literature, and no requirement for costly equipment. However other factors, including electrostatic interactions, temperature, and pH, can affect the results of these experiments. Contact angle (CA) data is affected, to a lesser degree than MATH assays by similar factors and by the requirement, when using specific equipment that the cells remain flat, and are dry.

The advantage of CA data is the multitude of information that can be generated from one set of data. To obtain contact angle measurements, a drop of liquid is deposited onto a given solid an image of the drop is projected onto a screen and a tangent is drawn close to the drop’s surface, at the liquid/solid/gas interphase. The angle at the solid/liquid/gas interface (θ) is called the contact angle (CA) (Fig. 3-1).

![Fig. 3-1. Contact angles (θ) are formed at the interface between the liquid, solid and gas boundaries of a droplet on the surface of interest.](image-url)
The contact angle ($\theta$) is a measure of how well a liquid wets a surface. An angle of $0^\circ$ indicates complete wetting, while an angle of $180^\circ$ indicates absolute non-wetting. More generally when contact angles are $< 90^\circ$ the liquid is able to wet the surface to some degree, and the surface is hydrophilic. Angles $> 90^\circ$ indicate that the surface is hydrophobic. At $90^\circ$ the surface energy of the solid is equal to the cohesive energy of water. The surface energy parameters of the solid can be determined if multiple, fully characterized liquids are used to obtain contact angles.

According to van Oss et al. (1990), this information can be used to obtain solid-liquid-solid interfacial energy ($\Delta G_{iwi}$), a measure of how quickly the surfaces will aggregate in liquids, when the solute is water; this is a measure of the hydrophobic nature of the surface. The surface energy parameters for the solid of interest are used to calculate $\Delta G_{iwi}$ by applying Young’s equation (Eq. 3-2) to the contact angles of multiple, well-defined liquids present on its surface.

$$\gamma_{SV} - \gamma_{SL} = \gamma_{VL} \cos \theta \quad (3-2)$$

Other surface energy parameters, such as the work of adhesion, the energy associated with the adhesion of a solid and a liquid, also defined as the work required to separate them (Eq. 3-2). The work of cohesion, the energy required to separate a liquid into two parts, can also be calculated from contact angle data using the following equations (Eq. 3-3 & 3-4).

$$W_a = \gamma (1 + \cos \theta) \quad (3-3)$$

$$W_c = 2\gamma \quad (3-4)$$

Where, $W_a =$ work of adhesion, $W_c =$ work of cohesion $\gamma =$ surface tension (liquid)/surface energy (solid)
It is no longer acceptable to use a single, static contact angle measurement to
determine surface energy properties, or wet-ability. Dynamic contact angles are
measured either while the drop is actively moving (advancing and receding angles), by
gradually increasing the volume of the drop, or by tilting the surface until the drop moves
while continuously measuring the advanced and receded angle. The advanced and receded
angles are defined as the angles obtained just prior to movement of the droplet. The
advanced/advancing angle is the angle that is formed just as the drop is about to move
over a dry surface. The receded/receding angle is the angle that is formed over already
wet surfaces. The advanced angle is less affected by hysteresis than the receded angle, so
it is the one most often reported (31, 86, 87).

Hysteresis, the difference between the advanced/advancing and the
receded/receding angles, is affected by surface homogeneities larger than approximately
10 nm and by hydrophilic and hydrophobic microenvironments on solid surfaces.
Hydrophobic micro domains are thought to have a breaking effect on the advanced/ing
angle, while hydrophilic domains appear to have a retarding effect on the receded/ing
angle. Sample roughness at the microscopic level increases hysteresis and has an effect
on hydrophobicity. Rough surfaces, that are otherwise identical to smooth surfaces, tend
to be more hydrophobic, especially if they already have hydrophobic characteristics
hysteresis (56).

While acid/base, dispersive and hydrophobic interactions can be calculated from
contact angle data, zeta potentials are the most effective way of determining the
electrostatic contribution. The movement of cells in an electrical field is directed by the
potential at the boundary between the cell and the ions tightly associated with it and the
surrounding medium. This potential is known as the zeta potential, and is a function of the cell’s surface charge, absorbed layers on the surface of the cell and the medium in which the cell is suspended. It is usually the same sign as the actual charge at the surface of the cell. The zeta potential is determined by measuring the movement of the cells in an electric field, or the particle mobility (velocity under unit field strength). There is a simple relationship between particle mobility (µ) and zeta potential (in water @ 25°C zeta potential = 12.85 µ).

**Materials and Methods**

**Cultivation of Fungal Cells**

*B. bassiana* (ATCC 90517) was routinely grown on potato dextrose agar (PDA). Plates were incubated at 26°C for 10-15 days, and aerial conidia were harvested by flooding the plate with sterile dH₂O. Conidial suspensions were filtered through several layers of Mira, cloth and the final spore concentrations were determined by direct count using a hemocytometer. Blastospores and submerged conidia were grown by inoculating conidia harvested from plates to a final concentration of 0.5 to 5 x 10⁵ conidia/ml into Difco™ Sabouraud dextrose (Becton, Dickinson and Co., MD) with 1-2 % yeast extract (SDY) and TK1 broth (83), with fructose as the carbon source, respectively.

Cultures, grown for 3 to 5 days at 26°C with aeration (150-200 rpm), were filtered (2X) through glass wool to remove mycelia. Final filtered suspensions of the three cell types were harvested by centrifugation (10,000 x g, 15 min, 40°C), washed twice with sterile dH₂O, and resuspended to the desired concentrations in appropriate buffers. Pre-centrifugation, post-centrifugation, and final spore concentrations were confirmed by direct cell count (10⁶-⁹ cells/ml).
Atomic Force Microscopy

Atomic force micrographs were generated using Digital Instruments’ Multimode SPM Atomic force Microscope with a silica nitrate tipped cantilever. Data from the micrographs was analyzed using Nanoscope SPM v.4.4 (Digital Images) and SPM Image Magic demo v1.10 (Alexander Kryzhanosvsky, A.F. Loffe physio-technical institute, St. Petersburg, Russia).

Zeta Potential

Aerial conidia, blastospores and submerged conidia were washed (3X) in water. The rinsed pellets were resuspended in 1mM KCl (pH 3-10, adjusted just prior to running the samples) to a concentration of $10^6$ cfu/ml within 1 hr of running the samples on a Zetaplus micro-electrophoretic unit (Brookhaven Instruments Cooperation) with native software (PALS). Each cell type was run in triplicate. Ten microphoretic readings were run for each triplicate for a total of 30 readings for each sample. The readings for the triplicate runs were averaged, and the standard error of the mean was determined for each sample.

MATH Assay

Cell surface hydrophobicity was determined essentially as described by Thomas et al. (1986). Briefly aerial conidia, blastospores, and submerged conidia were washed into PUM buffer (1.0 M sodium phosphate-urea-magnesium, pH 7.1). Fungal cell suspensions were adjusted to an $OD_{470} = 0.4$ and dispensed (3 ml) into acid washed glass tubes (12 x 75 mm). Hexadecane (300 µl) was added, each tube was vortexed (3 x 30 s) and allowed to incubated at RT for 15 min before the hexadecane phase was carefully removed and discarded. Tubes were cooled to 5°C and residual solidified hexadecane was removed. The tubes were warmed to RT, and the absorbencies of the resultant cell
suspensions were determined at 470 nm. The hydrophobic index (HI) was calculated using the following equation (Eq. 3-5):

\[ HI = \frac{\text{initial } OD_{470} - \text{final } OD_{470}}{\text{initial } OD_{470}} \]  

(3-5)

**Contact Angle Determination**

Contact angle measurements of the *B. bassiana* cell types were determined from digital images of drops obtained using a Ramé-hart Instruments Co. (NJ) Model 500 Advanced goinometer (automated drop dispenser, tilting plate, digital camera, native Dropimage Advanced software). Advancing angle measurements were determined just prior to movement of the water drop. Briefly, a 10 µl drop of solution was placed onto the surface of the substrata to be tested. The stage and the camera were tilted at 10 increments until the drop began to move. The leading edge (dynamic) contact angle was determined as the angle obtained just prior to drop movement.

Interfacial energies of interaction were calculated using the Acid/Base tool of the Dropimage Advance software. Water (polar), α-bromonapthalene (non-polar) and glycerol (polar) were used to determine the free surface energy components of the solid.

**Results**

**Atomic Force Microscopy**

Fascicle bundles (a rodlet layer) were clearly visible on *B. bassiana* aerial conidia, but were absent from both blastospores and submerged conidia (Fig 3-2.) The blastospore surface appeared smooth, whereas the submerged conidial surface was rough and a raised lip was apparent on most of the latter cells. No fascicles were visible on the germ tubes of germinating aerial conidia (data not shown).
Fig. 3-2. Atomic force micrographs of *B. bassiana* A) conidia, B) submerged conidia and C) blastospores. (bar A) 10 nm, B) 0.1 µm and C) 0.5 µm)

**Zeta Potential**

The surface charge of the three cell types decreased as the pH increased. The isoelectric point for all cell types was PI4, except 16-day-old conidia, which was PI5. Mean zeta potential values for aerial conidia, blastospores, and submerged conidia were obtained over a pH range from 3-9 (Fig. 3-2). These values were obtained for a minimum of 3 different samples; each sample was run at least 10 times. Aerial conidia (16 and 20-day-old combined) had a positive zeta potential (22 ± 2 mV) that rapidly became negative by pH 4-4.5, reaching a net negative surface charge of – 47 ± 4 mV at pH 6-7. Blastospores and submerged conidia also displayed a net positive zeta potential at low pH that decreased to – 4 ± 4 mV and – 13 ± 2 mV at pH 6-7, respectively (Fig. 4-3).

**MATH Assay**

Hydrophobicity indices (HI) indicated that almost 90% (HI= 0.88) of the aerial conidia partitioned into the hydrocarbon layer. Submerged conidia partitioned at an intermediate level (HI = 0.7), and at least 60% of the blastospores (HI = 0.4) partitioned preferentially in the aqueous phase. These values can be interpreted as relative hydrophobicity. However, absolute determination of which cell types are definitely hydrophobic and which are hydrophilic are not possible and the cutoff is often set arbitrarily at 70% (HI = 0.7) (66).
Fig. 3-3. Zeta potential values for the three spore types as a function of pH: (x) Conidia 16 days old, (∆) conidia, 20 days old, blastospores (♦), and submerged conidia (■).

Fig. 3-4. Microbical adhesion to hydrocarbon and hydrophobicity indices for *B. bassiana* aerial conidia, blastospores and submerged conidia.


Contact Angles

A surface is considered wettable if the liquid tested forms angles smaller than 90°. Water contact angles for the conidia of all three *B. bassiana* phenotypes were well over 90° (Table 3-1), while that of the submerged conidia, and the blastospores were consistently below 90°.

The propensity of two surfaces to adhere to each other in water is calculated by the interfacial energy of interaction ($\Delta G^{IF}_{w}$) between the surfaces (in this case the surfaces of two similar spores) in water. The original parental 90517 appeared to have two distinct phenotypes, which once separated bred true (Puff, 90517p and Blue, 90517b). The $\Delta G^{IF}_{w}$ of conidia, regardless of phenotype, was negative (90517 –34.8 mJ/m², 90517p –22.3 mJ/m², and 90517b –22.8 mJ/m²), indicating that these spores are hydrophobic; whereas the submerged conidia and the blastospores, had $\Delta G^{IF}_{w}$ values greater than 0 mJ/m² (30.2 mJ/m² to 46 mJ/m² and for submerged conidia and 66.3 mJ/m² to 75.2 mJ/m² for blastospores) indicating that they were hydrophilic (Table 3-2).

The values for acid/base component of the interfacial energy, $\Delta G^{AB}_{w}$ for conidial cells ranged from 23.9 mJ/m² to 69.4 mJ/m², are were below +102 mJ/m² ($\Delta G^{AB}_{w}$) which means they would be unable to overcome the cohesive energy of water dissolve in water. The values for blastospores and submerged conidia were all greater than +102 mJ/m²; specifically, 177.4 mJ/m² to 191.6 mJ/m² for the blastospores and, 171.5 mJ/m² to 212 mJ/m² for the submerged conidia indicating that these cells tend to disperse in water and are therefore hydrophilic. For the $\Delta G^{AB}_{w}$ value to be greater than 102 mJ/m², the electron donor surface energy parameter ($\gamma_S^+$) must be much lower than 25.5 mJ/m² ($\gamma_L^+$ for water), often around zero, and the proton acceptor surface energy parameter ($\gamma_L^-$)
must be much larger than 25.5 mJ/m² ($\gamma_L$ for water). This was found to be true for the blastospores and mycrocycle conidia, but not for the aerial conidia (Table 3-2).

**Discussion**

The surface properties of the three *B. bassiana* cell types, as measured by AFM, contact angles, and the MATH assay, were markedly different. Freeze-etch, carbon-platinum transmission electron microscopy has previously shown the presence of a conidial rodlet layer on the dry aerial conidia of many hyphomycetes, including *Beauveria bassiana* (5, 68, 97). In this dissertation Atomic Force Microscopy confirmed the presence of this layer on the surface of our strain of *B. bassiana*. Aerial conidia were shown to be highly hydrophobic, especially when compared to the other spore types investigated.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>mean Contact angle</th>
<th>Hydrophobicity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>$\alpha$-Bromonapthalene</td>
<td>Glycerol</td>
<td>$\Delta G_{iw}^{AB}$</td>
</tr>
<tr>
<td>90517</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerial Conidia</td>
<td>120.3±2.5°C</td>
<td>66.2±0.5</td>
<td>112.7±0.9</td>
<td>47.2</td>
</tr>
<tr>
<td>Blastospores</td>
<td>23.25±1.8</td>
<td>33.6±0.4</td>
<td>59.4±1.8</td>
<td>187.4</td>
</tr>
<tr>
<td>Submerged conidia</td>
<td>30.6±1.0</td>
<td>56.4±0.8</td>
<td>53.6±1.1</td>
<td>167.1</td>
</tr>
<tr>
<td>90517p&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerial Conidia</td>
<td>122.1±0.6</td>
<td>47.2±0.6</td>
<td>108.2±1.3</td>
<td>49.3</td>
</tr>
<tr>
<td>Blastospores</td>
<td>33.2±1.0</td>
<td>33.6±1.4</td>
<td>61.4±0.4</td>
<td>171.2</td>
</tr>
<tr>
<td>Submerged conidia</td>
<td>28.3±1.3</td>
<td>29.5±0.4</td>
<td>42.3±1.7</td>
<td>152.8</td>
</tr>
<tr>
<td>90517b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerial Conidia</td>
<td>119.3±0.7</td>
<td>42.6±0.4</td>
<td>122.0±1.4</td>
<td>50.0</td>
</tr>
<tr>
<td>Blastospores</td>
<td>23.9±0.8</td>
<td>39.5±0.5</td>
<td>68.9±0.9</td>
<td>183.7</td>
</tr>
<tr>
<td>Submerged conidia</td>
<td>28.6±1.0</td>
<td>38.8±2.7</td>
<td>40.8±1.7</td>
<td>147.3</td>
</tr>
</tbody>
</table>

a) Mean of advanced contact angles $n = 2 \times 8$ different samples (>2 drops were used per sample)

b) $\Delta G_{iw}^{AB}$ = acid/base, $\Delta G_{iw}^{IF}$ = interfacial energy.

c) Advanced/receded angles were measured just before the drop started to roll after the stage was tilted.

d) p (puff) and b (blue) two stable phenotypes, derived from the parent strain (90517)
Table 3-2. Advancing and receding water contact angle data with calculated surface energy values for three B. bassiana single cell propagules.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>(Y_{s}^{\text{AB}}) (Polar)</th>
<th>(Y_{s}^{\text{LW}}) (Dispersive)</th>
<th>(Y_{s}^{\text{total}})</th>
<th>Polar component</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(\gamma_{i}^+)</td>
<td>(\gamma_{i}^-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>90517</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerial Conidia</td>
<td>9.7±1.5</td>
<td>33.3±0.6</td>
<td>43.0±1.1</td>
<td>4.3±0.4</td>
</tr>
<tr>
<td>Blastospores</td>
<td>10.8±2.3</td>
<td>23.6±0.3</td>
<td>34.4±1.3</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Submerged conidia</td>
<td>23.3±1.1</td>
<td>16.3±0.4</td>
<td>39.6±0.8</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td><strong>90517p</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerial Conidia</td>
<td>0.6±0.01</td>
<td>33.3±0.3</td>
<td>33.9±0.1</td>
<td>9.4±0.5</td>
</tr>
<tr>
<td>Blastospores</td>
<td>3.2±0.8</td>
<td>24.5±0.50</td>
<td>27.7±0.7</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>Submerged conidia</td>
<td>16.6±2.0</td>
<td>25.1±0.30</td>
<td>41.7±2.5</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td><strong>90517b</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerial Conidia</td>
<td>13.6±1.1</td>
<td>30.3±0.2</td>
<td>43.9±0.7</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>Blastospores</td>
<td>7.3±1.4</td>
<td>21.7±0.6</td>
<td>29.0±1.0</td>
<td>0.16±0.1</td>
</tr>
<tr>
<td>Submerged conidia</td>
<td>24.5±0.9</td>
<td>24.7±0.4</td>
<td>49.2±0.8</td>
<td>2.8±0.4</td>
</tr>
</tbody>
</table>

a) AB = acid/base, s=solid, l= liquid gamma= surface energy components.
b) p (puff) and b (blue) two individual phenotypes, genetically identical to the parent strain (90517).

In 1999, Jeffs et al. concluded that the hydrophilic spores had more electronegative groups than the hydrophobic ones because amine modified, electropositive, polystyrene beads attached better to hydrophilic aerial conidia than to hydrophobic aerial. Our results suggest that the most hydrophobic spores, aerial conidia (based on zeta potential measurements) have more electronegative groups than the hydrophilic ones, but that the stronger, and thus predominant, interactions for these spores are hydrophobic and the electronegativity of the spores probably has a limited on effect initial interactions, although surface charge may be important for binding once the aerial conidia have made contact.

Blastospores, the most hydrophilic spores, have fewer electronegative groups, and electrostatic interactions appear to be the most important factors involved in surface interactions for these cells. Submerged conidia are intermediate in both hydrophobicity
and electronegativity, indicating that for these cells both factors are likely to play an important role in adhesion to solid surfaces, dispersion in liquids, and flocculation.

Previously, surface charge measurements of fungal spores often relied on indirect techniques, or were carried out at a single pH value. While these experiments yielded valuable information about the adhesion profiles of the spores, they often did not take into account the interactions of competing and synergistic forces on the adhesion profiles. As a result, indirect measurements of surface potential should not be the only criterion used when making inferences about the actual electric potential at the cell surface of the spores.

In these experiments, the electronegative character of aerial conidia increased steadily with pH, reaching values as low as –47 mJ/m² at pH 8.0. St. Leger et al. (1989) observed, that during fungal entomopathogenic infections, the pH of the insect cuticle rises from 6.3 to 7.7 during the initial phases, indicating that conidia will be moderately electronegative during the first stages of infection, but that they will become more electronegative as the infection progresses.

Because hydrophobic interactions are stronger and act over much larger distances than electrostatic forces, it is likely that the primary intermolecular interaction involved in the initial adhesion process is hydrophobic in nature. It is equally probable that other intermolecular interactions become relatively more important once the spore is in close proximity with the insect cuticle. The variety of intermolecular forces are involved in the adhesion process probably ensure that B. bassiana aerial conidia remain attached to the cuticle until the germ tube penetrates, despite rapidly changing micro-environmental conditions.
B. bassiana spores can vary widely in hydrophobicity, between strain as well as within the same strain, depending on age of the cells and culture conditions. This phenomena has been linked to possible specialization of the fungus towards different strains (51). Rodlet layers, while important to the overall hydrophobicity of fungal spores, are not the only cell surface features involved in hydrophobic interactions. Removing these layers decreases, but does not eliminate the hydrophobic character of Aspergillus fumigatus conidia, and has different effects on cell surface characteristics such as surface charge and ability to bind to hydrocarbons on A. nidulans and A. fumigatus (39).

Submerged conidia are similar in shape to aerial conidia, but are typically larger and do not appear to posses a highly organized rodlet layer (9). These cells (when imaged using AFM) appeared rough compared to blastospores whose surfaces appeared smooth. Previous reports have shown that if these cells do posses a rodlet layer it is highly disorganized (10, 83). Submerged conidia also had smaller absolute zeta potential than aerial conidia at higher pH. Although the environmental condition that leads to the formation of submerged conidia has not been well characterized, it has been observed that these cells are able to initiate infection at rates equal to or higher than aerial conidia (80, 83). Submerged conidia appear to be less tolerant to environmental stresses than aerial conidia and may be a specialized strategy related to nutrient/host availability.

Blastospores, the smoothest of the three single cell propagules, did not posses a rodlet layer and were the most hydrophilic of the spore types. Hyphal bodies present inside the insect during infection are similar in shape and size to these spores but appear to have much thinner cell walls than blastospores (71). The electrostatic properties of
blastospores did not vary as dramatically with pH as the other spore types, although previously we have noticed that the binding of these cells to hydrophilic surfaces is dependent on pH.

The ability of blastospores to attach under favorable conditions increased with increasing pH until the isoelectric point was reached (around pH 4). Adhesion did not however decrease with increasing pH >4.0 as expected, considering increased electrostatic repulsion under these conditions. Instead, the adhesion rates continued to increase as the ambient pH increased. As noted by Jeffs et al. (1999), this is probably due to other intermolecular factors stabilizing the adhesive process and minimizing the electrostatic repulsion. This may also explain why these spores have moderate electrostatic properties, compared with aerial conidia. Strong electronegative surface charge and weak hydrophobicity may result in increased electrostatic repulsion leading to decreased adhesion.

Both the MATH assay and contact angle data can be used to measure hydrophobicity. However, they do not give exactly the same information. In the MATH assay, the propensity of the spores to partition in one layer preferentially over another is used to assess the hydrophobic nature of the spores. However, this assay does not take into account other non-specific forces that may affect partitioning and as a result should be used as a direct measure of the surface hydrophobicity of the cells. Contact angles can give more precise determinations of the cell surface hydrophobicity (CSH); however the cells are usually dry when tested and this needs be taken into account when considering the results. Given these caveats, our results demonstrate that the three spore types are very different in terms of their surface characteristics.
Previous studies had conflicting results regarding the hydrophobicity of submerged conidia. Some reports noted that they were as hydrophobic as aerial conidia (83), but Jeffs et al. (1999), using SAT, noted that blastospores were less hydrophobic than aerial conidia. Our results appear to back up this last interpretation; however it should be noted that there appears to be great heterogeneity between the cell surface characteristics of the same type of spores from different *B. bassiana* strains. In addition, the hydrophobicity of the spores can vary within a strain, depending on the age of the culture and the cultural conditions.

This report also notes the importance of studying cell surface characteristics (CSC) of any spore that might be considered for commercial applications, using a range of biochemical parameters. The rational is that the microenvironment the spores are likely to encounter on the surface of the insect cuticle may change rapidly as the infection proceeds.
Introduction

Virulence Factors

Deuteromycete entomopathogenic fungi usually infect their arthropod hosts through the cuticle. Infection is dependent on the fungus successfully adhering to and penetrating through the host’s cuticle. Once inside the host the fungi must avoid being destroyed by the immune response and in some cases have a means of exiting the host and dispersing its spores into the environment so that the cycle can continue. The molecular factors important to the infection process determine if an organism is pathogenic and which hosts it can invade, these factors are collectively termed virulence factors.

There are a number of definitions in the literature describing virulence factors. Some of the most common of these definitions include: 1) components that when deleted specifically impair virulence, but not the ability of the organism to grow, 2) products that allow the organism to cause disease and 3) components of the pathogen that cause damage to the host.

The factors involved in the pre-penetration and penetration steps are important for initiation of infection, but are often redundant, or they work in tandem with other virulence factors. This makes it difficult to define them as virulence factors under the first definition because it might be difficult to show that virulence is decreased when
candidate genes are deleted. In the case of adhesion factors, while they are important in initiating the infectious process, they often do not cause significant damage to the host tissue, making it difficult to define under the third definition of virulence factors requiring virulence factors to cause significant damage to host tissue.

The Edward Jenner institute for vaccine research lists the following groups of virulence factors, based on definitions in current literature, as follows: 1) adherence and colonization factors (e.g. adhesions, integrins, hydrophobins), 2) invasins (extracellular enzymes involved in breaking down host defenses, including proteases and chitinases), 3) toxins (e.g. cerato-plantatin and snodprot1), 4) molecules that permit the pathogen to evade the host’s immune system, 5) siderophores, and 6) molecules involved in the transport of toxins.

**Adherence and Colonization factors**

The adhesion of entomopathogens to the insect cuticle is mediated by specific and non-specific factors including adhesins (e.g. hydrophobins), integrins (molecules containing the tripeptide sequence arginine-glycine-aspartic acid (RGD) recognizable by molecules on host cells, lipids, and polysaccharides (84).

**Invasins**

Entomopathogenic fungi secrete specific extracellular enzymes (invasions) during pre-penetration and penetration events. The activity of infection specific proteases increases in the presence of insect cuticle and secretion is often regulated by environmental cues (pH and nutrients) that signal to the organism that it is on the cuticle of a suitable host. Many entomopathogenic fungi secrete substilin-like proteases (e.g. Pr1), and trypsin like proteases (e.g. Pr2) in response to environmental cues including high pH and nutrient deprivation ph (78). St. Leger et al. (1998) examined pH dependent
secretion of *M. anisopliae* proteases and noted that these enzymes (Pr1, Pr2 and metalloproteases) that are typically secreted in response to the presence of insect cuticle and at higher pH (pH 8) than aspartyl proteases and amino peptidases which are not typically involved in cuticle degradation. The highest activity of chitinases is observed when the fungi are exposed to chitin and is secreted later in the infection process than infection related proteases (78). The expression of hydrophobins implicated in adhesion and recognition of cuticle surfaces appears to be regulated by mechanisms and environmental cues similar to those that regulate the serine proteases and metalloproteases (78).

**Toxins**

Information relating to toxin production during infection comes mainly from the study of plant pathogenic fungi. During the infection process these fungi employ similar adhesion and penetration mechanisms as those observed in entomopathogenic fungi. The wheat pathogen *Phaeosphaeria nodorum* produces a hydrophobin-like protein (Snodprot1), which is secreted mainly during invasion. Although this protein accumulates at the site of infection and brown lesions that progressively increased in size can be observed at the site of application of drop diffusates from *phaeospheria nodorum* infected wheat leaves, the purified snodprot1 protein appears to have no specific cytotoxic effects (44). Conversely, purified extracts of a protein (cerato-platanin) highly homologous highly produced by the tabacco pathogen *Ceratocystis fimbriata* the causative agent of canker stain, shown to cause necrosis in tabacco leaves (69). Snodprot-like proteins are related to hydrophobins by n-terminal sequence homology, level of hydrophobicity, numerous cysteines and size.
Enzymes of pathogenic fungi involved in the synthesis of small cyclic polypeptides, or polyketide synthetases, are sometimes considered virulence factors. Polyketides are secondary metabolites produced by a variety of organisms including, fungi, plants, insects, and mollusks (60). Polyketides important for host-pathogen interactions include antibiotics (e.g. erythromycin A), anti-tuberculosis compounds (e.g. rifamycin B), anti-helmintic compounds (e.g. avermectin) and numerous immunosuppressant agents including ripamycin polyketides.

**Molecules Involved in Evading Host Defences**

Successful entomopathogenic infections depend on the pathogen evading the host’s immune response, and/or using this response to increase the spread of the organism through the organism. *Aspergillus* spp. have numerous virulence factors, the ones that have been implicated with evasion of the host’s immune system include the conidial rodlet layers, conidial melanins, detoxifying systems for reactive oxygen species (catalases and super oxide dismutases) (73).

**Siderophores**

Iron is a catalytic cofactor in many redox reactions; as a result it is an essential nutrient for many pathogenic fungi. Iron is relatively unavailable because it is typically found in the insoluble ferric form (oxides and hydroxides) and within the host it is usually bound to carrier proteins such as transferrin. Pathogenic organisms obtain iron from scavenging systems such as high affinity iron permeases, siderophores (iron specific chelators) or haem oxygenases which acquire iron from haem groups (67).

**Molecules Involved in Toxin Transport**

Shapiro-Ilan et al. (2002) examined the natural and induced variability of the resistance of *Beauveria bassiana* strains to fungicidal agents. They noted that exposure
to fungicides increased virulence against insect hosts, and this effect was observed without concurrent exposure of the fungicide to the insect. Indicating that being exposed to fungicides, in a manner not well understood, increased the organism’s virulence (76).

*Beauveria* bassiana strains are major secretor of toxins. Transporters are required to secrete these molecules and protect the organism from toxins produced by the host. There are two major groups of multidrug transporters the major facilitator superfamily (MFS) transporters and the ATP-binding cassette (ABC) transporters (21).

**Suppression Subtractive Hybridization**

Suppression subtractive hybridization (SSH) a technique developed by Diatchenko *et al.* (1996), combines normalization and suppression into a single procedure. Within a single subtractive cycle, SSH suppresses the amplification of a portion of abundantly expressed sequences, normalizing the ratio of high and low abundance transcripts. Differentially expressed transcripts are selectively amplified without the need to physically separate single stranded (SS) cDNA from double stranded (DS) cDNA (22).

Many proteins and enzymes involved in pre-penetration, and penetration stages are secreted in response to insect cuticle. The enzymes secreted at this stage are more efficient in breaking down insect cuticle than similar extracellular enzymes produced by non-pathogenic fungi, or by the entomopathogenic fungi growing saprophytically enzymes (13). It has been relatively difficult to correlate individual enzymes with virulence, or pathogenicity, and thus to conclusively defined them as virulence factors. This is often due to the high redundancy of these enzymes, many different isoforms of the same type of enzyme (e.g. Pr1 and pr1b, multiple chitinases, etc) that appear to replace those in mutant entomopathogens lacking specific hydrolases (13).
Materials and methods

Cultivation of Fungi

*Beauveria bassiana* (ATCC 90517) was routinely grown on Luria Bertani (LB) (Difco, MI) (4). Plates were incubated at 26°C for 10-14 days, and aerial conidia were harvested by flooding the plate with sterile dH2O. Conidial suspensions were filtered through a single layer of Mira-cloth (Clabiochem, CA) and resuspended in sterile deionized water. Final spore concentrations were determined by direct count using a hemocytometer. Czapek dox (Difco, MI) broth (24 ml) was inoculated with a loopful of the colonies on the LB agar plates and incubated at 26°C with aeration (210-230 rpm).

**Cells grown in the presence of glucose (source of driver RNA)**

Minimal medium with 1% (w/v) glucose (0.4 g/l KH$_2$PO$_4$, 1.4 g/l Na$_2$HPO$_4$, 0.6 g/l MgSO$_4$.7H$_2$O, 1.0 g/l KCl, 0.25 g/l NH$_4$NO$_3$, 0.01 mg/l FeSO$_4$ and 10 g/l glucose) was inoculated with 0.1 ml of the Czapek dox (24 ml) cultures (6 days). Cultures were grown for 6 days at 26°C with aeration (210-230 rpm) and filtered through a 0.22 µm filter (Corning, NY), to remove culture supernatant. The mycelial mat was washed (2x) with water and then lyophilized.

**Cells grown on insect cuticle (source of tester RNA)**

The insect cuticles were removed from frozen (-20°C) mole crickets (*Scapteriscus vicinus*). The cuticles were disinfected by soaking in them 30% ethanol (30 min), followed by 30% chlorox (2 x 15 min). The cuticles were rinsed repeatedly with water until there was no detectable chlorine odor.

Minimal medium with 1% (w/v) glucose (0.4 g/l KH$_2$PO$_4$, 1.4 g/l Na$_2$HPO$_4$, 0.6 g/l MgSO$_4$.7H$_2$O, 1.0 g/l KCl, 0.25 g/l NH$_4$NO$_3$, 0.01 mg/l FeSO$_4$ and 10 g/l sterilized cuticle) was inoculated with 0.1 ml of the Czapek dox (24 ml) cultures (6 days). Cultures
were grown for 6 days at 26°C with aeration (210-230 rpm) and filtered through a 0.22 µm filter (Corning, NY), to remove culture supernatant. The mycelial mat was washed (2x) with water and then lyophilized.

**RNA Isolation**

Fungal cells were frozen in liquid Nitrogen, or lyophilized and stored @ -70°C until used. Immediately prior to use the cells were crushed in liquid Nitrogen and total RNA was extracted with RNAwiz™ (Ambion, TX) per manufacturer’s instructions. Poly A RNA was enriched using magnetic beads covalently attached to oligo-dT tails (Dynabeads mRNA direct kit, Dynal, WI, Poly (A) Quick® mRNA isolation kit, Stratagene CA, or Poly A tract® mRNA isolation Systems, Promega, WI).

**Suppression Subtractive Hybridization**

The PCR-Select cDNA subtraction kit (Clontech, CA) used as per manufacture’s instructions. The RNA purified from cells grown on glucose was designated as the driver RNA, while the RNA from cells grown on insect cuticle, was designated as the tester RNA. The amplified PCR product was cloned into Topo® TA (Invitrogen, CA) or pGEM® -T easy (Promega, WI) cloning vectors.

**Results**

Approximately 350 clones were sequenced and analyzed, resulting in 280 non-redundant sequences. Of these, 57% had no homology to known proteins, had weak homology (expect value < 1e-5) to known proteins, or gave hits to hypothetical, or proteins of unknown function. The remaining proteins were loosely grouped according to function: (i) Enzymes (10%), (ii) Miscellaneous (proteins that did not fit neatly into specific groupings (18%), (iii) signal transduction (6%), (iv) virulence factors (1%), (v)
allergens (2%), and (vi) ribosome/histone proteins (5%) (Fig. 4-1). The proteins with weak homology to specific functional groups were listed accordingly in figure 4-1.

Fig. 4-1. Relative numbers of gene fragments representing functional groups present in the subtracted library, the smaller graph represents proteins with no homology to known proteins, strong homology to hypothetical proteins, or proteins with unknown function or with weak homology (expect value < 0.0001) to proteins with known functions.

Among the virulence factors, two fragments were sequenced that gave strong hits (4e⁻⁷ and 2e⁻⁶) to a class I, rodlet layer hydrophobin belonging to the *Aspergillus* genus (expect values for whole genes: 2e⁻³⁶ *A. fumigatus* (rodA), 1e⁻³⁴ *A. nidulans* (rodA) and 4e⁻³⁰ *Gibberella moniliformes* (HYD1)). In addition to the hydrophobins, strong hits were obtained to a tetracycline efflux protein (1e⁻¹⁵) an allergen (9e⁻⁵) (Table 4-1). The full expressed and genomic sequence for *bhd1* was obtained (Keyhani, unpublished results) (fig. 4-2) and this information was used to design the primers for the real time RT-PCR.
Table 4-1. Blast hits of sequence fragments from the SSH library to virulence factors and allergens.

<table>
<thead>
<tr>
<th>ID</th>
<th>function</th>
<th>e value</th>
<th>type</th>
<th>Known virulence factor</th>
<th>accession #</th>
</tr>
</thead>
<tbody>
<tr>
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<td>angi_emeni antigen 1 precursor</td>
<td>9.00E-05</td>
<td>allergen</td>
<td></td>
<td>emb</td>
</tr>
<tr>
<td>1RCA-F05.g-b1</td>
<td>Tetracycline efflux protein</td>
<td>1.0E-15</td>
<td>antibiotic transport</td>
<td>x</td>
<td>emb</td>
</tr>
<tr>
<td>2-4-E02.g-b1</td>
<td>rodl_emeni rodlet protein</td>
<td>2.00E-06</td>
<td>hydrophobin</td>
<td>x</td>
<td>sp</td>
</tr>
<tr>
<td>1RCA-A06.g-b1</td>
<td>Rodl_emeni rodlet protein precursor</td>
<td>4.0E-07</td>
<td>hydrophobin</td>
<td>x</td>
<td>sp</td>
</tr>
<tr>
<td>1RCA-B06.g-b1</td>
<td>STARP antigen</td>
<td>1.0E-06</td>
<td>invasin</td>
<td></td>
<td>dbj</td>
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Discussion

Our subtracted library generated 10 putative virulence factors (including B. bassiana hydrophobin, bhd1, a tetracycline efflux transporter and two potential allergens (STARP and ASPII)), however we were unable to find a number of expected virulence factors including serine proteases and chitinases that are secreted during the infection stage. This is probably due to the mixture of cells present in the library, many of which may not have been producing proteases, and to the lack of synchronization or the cells with resect to the infection process.
In our laboratory we have generated a series of *B. bassiana* stage and cell specific cDNA libraries (Cho et al personal communication). From these libraries it has become apparent that many of these proteins are expressed under conditions present in our driver library, and that further analysis of subtracted libraries of purified cells from different stages would give a better picture of the expression of putative virulence factors.

The fragments hydrophobin (*bhd1*) and other potential virulence factors found in the SSH library were sequenced, cloned and the full-length sequences were obtained using 5’prime RACE (Keyhani, unpublished results) when needed (some of the smaller genes did not require additional analysis to generate full length cDNA sequences). PSI-Blast searches indicated that the hydrophobin had the strongest homology to the RodA proteins of *A. fumigatus* and *A. nidulans*, and a weaker homology to the HYD1 and HYD2 proteins of *Gibberella moniliformis* (anamorph: *Fusarium verticillioides*).

However, Bootstrapped, phylogenetic analysis (Phylip 3.6, ClustalX, and phylodendron) of class I and class II hydrophobins deposited in the NCBI protein database, from *Ascomycetes* and the *Basidiomycetes*, places them closer to the *F. verticillioides* hydrophobins (figures 4-3 & 4-4).
Fig. 4-3. Alignment of H1 and H2 with homologous hydrophobins (ClustalX) that gave significant expect values in PSI blast. Signal peptide length was determined using SignalP.
Fig. 4-4. Bootstrapped (1000) phylogenetic tree of class I and class II hydrophobins using alignments generated with ClastalX and visualized with the online tree printer Phylodendron (http://iubio.bio.indiana.edu/treepub/treeprintform.html)
This is interesting because the *F. vercticilliodes* microconidia are hydrophilic (38) and this organism is a plant pathogen with specificity towards corn and *B. bassiana*, an entomopathogen with hydrophobic conidia, can infect corn asymptptomatically (88). It is possible that some of the same factors that allow *F. vercticilliodes* to infect corn are similar to those that allow *B. bassiana* to colonize the same organism.

It is possible that the expected genes were present in the subtracted library. The fragments generated by the SSH procedure are quite small making it difficult to determine if the fragments did not have strong hits because there were not matches, or if they were to small to generate strong hits. Annotated information for fungal genomes lags behind that for many other organisms, and as more annotated sequences are deposited into the databases, more hits to named proteins is likely to occur.
CHAPTER 5
MOLECULAR ANALYSIS OF TWO BEAUVERIA BASSIANA HYDROPHOBINS
AND A HYDROPHOBIN LIKE PROTEIN

Introduction

Hydrophobins are small, cysteine rich, secreted proteins unique to fungi. Many hydrophobins were initially identified from abundantly transcribed mRNA present during sporulation, fruit body formation, and during pathogenic interactions with plants and animals (52). These proteins play a critical role in numerous filamentous fungal processes including; growth, conidial formation, conidial dispersion, aerial structure formation, and attachment to (hydrophobic) surfaces, and pathogenicity (93).

Wessels et al. (1991) noted a positive correlation between Sc3 mRNA abundance and the presence of aerial hyphae and fruiting bodies in Schizophyllum commune. They estimated that by the fourth day of growth up to 8.1% of the protein synthesis activities in the dikaryon (fruiting bodies are composed of dikaryon hyphae) is directed towards the synthesis of hydrophobins.

Rodlet layers have been observed on conidial surfaces of numerous filamentous fungi including, Neurospora crassa, Aspergillus sp., Magnaporthe grisea, Metharhizium. anisopliae and Beauveria bassiana. The presence of this rodlet layer appears to increase the hydrophobic nature of the conidia. The better organized, the fascicles, the more hydrophobic the spores. Hydrophilic conidia formed by fungi such as Gibberella moniliformis, Verticillium lecanii and Botrytis spp. do not appear to form a
rodlet layer, and have additional structures that mediate attachment to surfaces including mucosal coats, and adhesive knobs (94, 99).

Hydrophobins produced during sporulation are involved in the production of the rodlet layer that has been observed on the surface of many hydrophobic conidia. Deletion mutants lacking *rodAp* in *A. nidulans* and *A. fumigatus* (68) or *mpg1* in *M. grisea* (52) generated spores lacking the characteristic rodlet layer, and displaying altered adhesion characteristics. The relative importance of the hydrophobins to rodlet layer formation and adhesion properties may vary with species.

Paris *et al*. 2003 observed that both *A. fumigatus* and *A. nidulans* possess two conidial hydrophobins *RodAp* (both sp.) *RodB* (*A. fumigatus*) and *DewAp* (*A. nidulans*). Deleting *rodAp* resulted in loss of the conidial rodlet layer in *A. fumigatus* while deleting *rodBp* had no effect on rodlet layer formation. In *A. nidulans* similar studies showed that both *rodAp* and *dewAp* were required for conidial rodlet layer formation. Paris *et al*. (2003) also noted that changes in cell surface characteristics were more profound in the *A. nidulans* rodletless mutants than in the corresponding *A. fumigatus* mutants.

Unlike *A. nidulans* and *A. fumigatus*, the rodlet layer of *B. bassiana*, *Neurospora crassa* and *Magnaporthe grisea* appear to possess a single hydrophobin (68). In *M. grisea*, deleting *mpg1* resulted in rodletless mutants similar to those observed for *A. fumigatus* and *A. nidulans* (52). This protein was also linked to pathogenicity by the observation that appropriate appressorium formation, in response to external surface cues, was impaired in ∆*mpg1* mutants (3).

All hydrophobins isolated to date self-assemble into 10 nm thick layers at hydrophobic/hydrophilic interfaces. Based on the solubility characteristics of the layers
and hydropathy patterns of the amino acids, hydrophobins have been separated into two classes (I and II) (96). Treatments with trifluoroacetic acid (TFA), or formic acid (FA) are often required to dissolve class I layers which remain insoluble in 2 % sodium dodecyl sulfate (SDS) at 100°C. In contrast, layers formed by class II hydrophobins are more soluble and can be dissolved in 60 % ethanol and 2 % SDS.

Class I hydrophobins self assemble at hydrophilic/hydrophobic interfaces, forming 5-12 rodlets that bundle together into fascicles. The rodlets are made up of protofilaments 2.5 nm in diameter. Thus far, similar bundles have not been observed in the amphipathic layers formed by Class II hydrophobins (96). Atomic resolution of the structure of HfbI and HfbII, hydrophobins from *Trichoderma reesei*, revealed that these Class II hydrophobins form tetrameric supramolecules that assemble into crystalline domains (43).

The rodlet layer, like the proteins from which it is composed, is amphipathic. When coating the surface of fungal structures this layer arranges itself so that the hydrophilic part of the membrane faces the cell wall of the fungus, while the hydrophobic side is exposed to the environment. In *A. nidulans* and other fungi with hydrophobic conidia this layer, especially when present on the surface of conidia, is thought to be important in hydrophobic interactions between fungus and surfaces, however in the closely related species, *A. fumigatus* adhesion to hydrophobic substrates is not completely lost in mutant strains lacking the major spore coat hydrophobins (68).

There is little sequence homology between Class II hydrophobins, and even less for Cass I hydrophobins, although all hydrophobins share eight cysteines that are characteristic of these molecules. Complementation studies of rodlet hydrophobins from
closely related species, such as *A. nidulans* and *A. fumigatus*, show that they often can restore partial wild-type characteristics, however when the fungi that are not as closely related, as is the case with *M. griseae* and *A. nidulans*, phenotypes may not be restored unless the gene is introduced under the control of the promoter from the host organism’s own hydrophobin suggesting that regulation may be more important than the hydrophobin’s sequence in determining function (52, 97, 98).

In addition to being the major proteins in the rodlet layer of hydrophobic conidia, hydrophobins are also found on the surface of hyphae and fruiting bodies. They are highly surface active molecules and reduce the surface tension of water allowing the fungus to form aerial structures (62). They have also been implicated in signaling (82) and are important for mediating adhesion. Determining the mRNA levels of *bhd1, bhd2* and *bsn* in different cell types and under different cultural conditions can be expected to yield insights into their function.

In this study real-time reverse transcriptase PCR (real-time RT-PCR), currently the most sensitive technique for mRNA detection was used to quantify the levels of transcripts encoding the two hydrophobins (*bhd1* and *bhd2*) and the hydrophobin like protein (*Bsn*) in aerial conidia, submerged conidia and blastospores.

**Materials and Methods**

**Cultivation of Fungi**

*Beauveria bassiana* (ATCC 90517) was routinely grown on potato dextrose agar (PDA) (4). Plates were incubated at 26°C for 10-14 days, and aerial conidia were harvested by flooding the plate with sterile dH₂O. Conidial suspensions were filtered through a single layer of Mira-cloth (Clabiochem, CA), and final spore concentrations were determined by direct count using a hemocytometer. Blastospores were produced in
Difco™ Sabouraud dextrose (Becton, Dickinson and Co., MD) + 1-2% yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of 0.5 - 5 x 10⁵ conidia/ml as the inoculum. Cultures were grown for 3-4 days at 26°C with aeration (150-200 rpm). Cultures were filtered (2x) through glass wool to remove mycelia, and the concentration of blastospores was determined by direct count. Submerged conidia were produced in TKI broth using fructose as the carbon source as described (83). For all cell types, Mira-cloth (Clabiochem, CA) or glass wool filtered cell suspensions were harvested by centrifugation (10,000 x g, 15 min, 4°C), washed two times with sterile dH₂O, and resuspended to the desired concentration as indicated (typically 10⁷-10⁸ cells/ml). Cells producing oosporein were grown in Difco™ Sabouraud dextrose (Becton, Dickinson and Co., MD) + 1-2% yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of 0.5-5 x 10⁵ conidia/ml as the inoculum until they started producing oosporein, and the mycelium were obtained from unfiltered cultures used to grow blastospores.

**RNA Isolation**

Fungal cells were frozen in liquid Nitrogen, or lyophilized and stored @ -70°C until used. Immediately prior to use the cells were crushed in liquid Nitrogen and total RNA was extracted with RNAwiz™ (Ambion, TX) per manufacturers instructions. Poly A RNA was enriched using magnetic beads covalently attached to oligo-dT tails (Dynabeads mRNA direct kit, Dynal, WI, Poly (A) Quick® mRNA isolation kit, Stratagene CA, or Poly A tract® mRNA isolation Systems, Promega, WI).

**Rodlet Layer Extraction**

The rodlet layer proteins were removed from the surface of the spores as described by Paris _et al._ (68). Briefly, aerial conidia, blastospores, and submerged conidia were
prepared as described above, resuspended in water, and sonicated at 140 W (3-mm diameter microtip, 50% duty cycle) for 2 x 10 min using a Sonifier cell disrupter B-30 (Branson Ultrasonics, Rungis, France). Unlysed cells and cell debris were removed by low-speed centrifugation (10,000 g 10 min), and the supernatant was centrifuged for 1 hr at 50,000 g. The resultant pellet was boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol in 62 mM Tris-HCL, pH 6.8), washed twice with equal volumes of sample buffer then three times with equal volumes of distilled water. The final pellet was lyophilized, then treated with 100% trifluoroacetic acid (TFA), and incubated for 10 min at room temperature. The acid was removed under a stream of nitrogen, and dried extracts were stored at room temperature under dry air. The sample was resuspended in water prior to analysis.

Aliquots of protein sample were mixed with lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, CA) plus dithiothreitol (DTT) (NuPAGE reducing agent, Invitrogen, CA) separated by 10-12% Bis-Tris NuPAGE gel electrophoresis by a MES-SDS gel running buffer (Invitrogen, CA). Presumed protein molecular mass was estimated using SeeBlue® Plus2 Pre-Stained Standards (Invitrogen, CA). Protein bands were visualized with Sypro-Ruby Red (Biorad, CA) and coomassie blue staining.

**Mass Peptide Spectrometry (Peptide Fingerprinting)**

Samples were analyzed by the Protein Core facility (ICBR) at the University of Florida. Basic procedure: Proteins separated by SDS gel electrophoresis were cleaved in situ with trypsin. MALDI-MS was used to obtain a mass spectrum of the peptide
mixture. The resulting amino acid sequences were compared to the predicted sequences of Bhd1, Bhd2 and Bsn.

**Reverse Transcriptase RT-PCR**

Primers were designed to amplify sequences 80-275 bp in length within the Hydrophobin 1, Hydrophobin 2, Snodprot and the *B. bassiana* β-tubulin sequences. Primers are listed in Table 4-1.

**Table 5-1. Primer sequences and product size for the reverse transcriptase RT-PCR**

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Primer Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobin 1</td>
<td>F: ATC ATG CGT TTC GCT CTT GCC ATC</td>
</tr>
<tr>
<td></td>
<td>R: AGG TGG GAA AGA AGA CCA TCA GCA</td>
</tr>
<tr>
<td>Hydrophobin 2</td>
<td>F: AAA TGC TTG CCA CCA CCA TCT TCG</td>
</tr>
<tr>
<td></td>
<td>R: CTG CTG CTT GCA CTT GTT GTC GAT</td>
</tr>
<tr>
<td>Snodprot</td>
<td>F: TTC CGC GGT TAG AGT TTC TGG GGA</td>
</tr>
<tr>
<td></td>
<td>R: ACC CTG CTT CTG ATA CTG GGG CAT</td>
</tr>
<tr>
<td>Tubulin</td>
<td>F: TCC TTC GTA CGG TGA CCT GA</td>
</tr>
<tr>
<td></td>
<td>R: CGA GCT TGC GAA GAT CAG AG</td>
</tr>
</tbody>
</table>

Total RNA concentration was determined with Molecular Probe’s (OR) Ribogreen® RNA quantitation kit, per manufacturer’s instructions. Plasmids containing the *hyd1, hyd2 and bsn* sequences were used to generate standard curves to determine the original concentration of the RNA amplified from the experimental treatments and to ensure that all genes were being amplified at the same rate. Assays were run in triplicate, in 96 well plates, a Gemini XPS system fluorescent plate reader (Molecular devices, CA) running native Soft Max Pro®, software. Appropriately diluted samples (pg to ng quantities) were amplified using Biorad’s iScript One-Step RT-PCR kit with SYBR green, on Biorad’s iCycler with it’s native software (Biorad, CA). The amplification cycle was as follows: 1 x 10 min @ 50°C, 1 x 5 min @ 95°C, and 45 x (10 sec @ 95°C and 30 sec @ annealing temp).
Results

Identification of a *B. bassiana* Cell Wall Hydrophobin

Four prominent bands (aprox. 25 KDa, 12 KDa and 4-5 KDa) were observed when SDS soluble/TFA insoluble proteins, from *B. bassiana* conidial cell walls, were subjected to SDS-PAGE (Fig. 5-1). It was postulated that the 12-14 KDa, TFA soluble/SDS insoluble protein isolated from the conidial cell wall was either *Bhd1* (12KDa) or *Bhd2* (14KDa). Peptide fingerprinting (peptide mass spectrometry) identified the sequences for two fragments of the 12-14 KDa band (Fig. 5-2 & 5-3). The sequence of fragments from the 12-13KDa band identified by mass spectroscopy were compared to the sequences of *Bhd1* and *Bhd2* which combined covered 29.2% of the amino acid sequence (35/116), or 30.9% (36991/11983.9) of the mass predicted for Bhd2 (Fig. 5-3).

![Fig. 5-1. SDS –PAGE (10% polyacrylamide, Bis-Tris) Gel of SDS soluble/TFA insoluble cell wall proteins stained with coomassie blue Lane 1, protein molecular weigh standards; lane 2, aerial conidia; lane 3, blastospores; and lane 4 submerged conidia.](image)

Reverse Transcriptase RT-PCR.

In addition to the sequence for *Bhd1*, the cDNA and genomic sequences for another *B. bassiana* hydrophobin (*bdh2*) and a *B. bassiana* hydrophobin like protein (*Bsn*) have been identified in our laboratory (E. Cho unpublished results). The *bdh1* mRNA levels
were highest in the submerged conidia with a relative concentration (RC) of 41.77. Aerial conidia and blastospores had much lower levels with RC 3.61 and 1.69 respectively.

The relative amount of \( bdh2 \) mRNA (a cell wall SDS insoluble/TFA soluble protein) was much lower than that of \( bdh1 \) in all cell types (RC 1.69 - 41.77 for \( bdh2 \) and RC 0.01-1.59 for \( bdh1 \)). The highest abundance of \( bdh2 \) mRNA was found in aerial conidia (RC 1.59) much lower levels were found submerged conidia (RC 0.18) and blastospores (RC 0.01). The concentration of the tubulin mRNA was lower in conidial cells ([\( tub \)/[total RNA] = 0.001), when compared with the other two spore types ([\( tub \)/[total RNA] = 0.02). We were unable to demonstrate high levels of expression in any of the cell types tested; we also were unable to find significant expression of Snodprot in any of the cell types analyzed (Table 5-2).

Table 5-2. mRNA abundance of \( bhd1, bhd2 \) in \textit{Beauveria bassiana} single cell propagules.

<table>
<thead>
<tr>
<th></th>
<th>Aerial conidia</th>
<th>Submerged conidia</th>
<th>Blastospores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>89.7 ng/ml</td>
<td>838.6 ng/ml</td>
<td>649 ng/ml</td>
</tr>
<tr>
<td>[( tub )] ng/ml</td>
<td>0.09</td>
<td>1.46</td>
<td>15.96</td>
</tr>
<tr>
<td>[( bhd1 )] ng/ml</td>
<td>0.33</td>
<td>61.02</td>
<td>26.97</td>
</tr>
<tr>
<td>[( bhd2 )] ng/ml</td>
<td>0.15</td>
<td>0.26</td>
<td>0.20</td>
</tr>
<tr>
<td>[( bhd1 )/[( tub )]</td>
<td>3.61</td>
<td>41.77</td>
<td>1.69</td>
</tr>
<tr>
<td>[( bhd2 )/[( tub )]</td>
<td>1.59</td>
<td>0.18</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a) Ribogreen assay (Molecular probes, Invitrogen, CA).
b) \( tub \) (tubulin gene), \( bhd1 \) (\textit{Beauveria bassiana} hydrophobin 1), and \( bhd2 \) (\textit{Beauveria bassiana} hydrophobin 2).
c) The concentrations were normalized using the concentration values obtained amplification of the tubulin genes.
Avg Mass: 11983.9
Seq #   b       y (+1)
--- --- ------   ------  --
L   1   114.1   1385.8  13
T   2  215.1    1272.7  12
G   3  272.2    1171.6  11
P   4  369.2    1114.6  10
S   5  456.2    1017.6  9
V   6  555.3    930.5   8
L   7  668.4    831.5   7
S   8  755.4    718.4   6
D   9  870.5    631.3   5
L  10 983.5     516.3   4
D  11 1098.6   403.2   3
L  12 1211.7   288.2   2
R  13 1367.8    175.1   1

Fig. 5-2. Mass spectroscopy data showing the mass of individual amino acids in one of the two main fragments from the 12 KDa trifluoroacetic acid soluble/sodium dodecyl sulfate insoluble *B. bassiana* cell wall protein.
Coverage: 13/116 = 11.2\% by amino acid count, 1385.6/11983.9 = 11.6\% by mass

Hydrophobin sequence H2
MLATTIFATL LALAAAAPHG PSHPGVKTG DICGNMTMH CCNDESVTNK LTGPSVSDDL DLRLHAAEC SPISVNLIN QLVPIDNCK QQSICCGEQK LNGLNLDGCT PTVLG

PepStat LTGPSVSDDLRL position: 51 - 63

Avg Mass: 11983.9
Coverage: 22/116 = 19.0\% by amino acid count, 2313.5/11983.9 = 19.3\% by mass

Hydrophobin sequence H2
MLATTIFATL LALAAAAPHG PSHPGVKTG DICGNMTMH CCNDESVTNK LTGPSVSDDL DLRLHAAEC SPISVNLIN QLVPIDNCK QQSICCGEQK LNGLNLDGCT PTVLG

PepStat TGDICGNMTMHCCNDESVTNK position: 29 - 50

Fig. 5-3. Mass peptide fingerprinting results for two identifiable fragments of the 12 KDa trifluoroacetic acid soluble, sodium dodecyl sulfate insoluble \textit{B. bassiana} cell wall protein confirming that this protein is the Bhd2 hydrophobin.

\section*{Discussion}

Fragments of the hydrophobin gene (\textit{bhd1}) present in the SSH library (chapter 4) were sequenced, cloned and were ultimately found to collectively represent the full-length sequence of the gene (Keyhani, unpublished results). Since then we have identified sequences for two additional proteins Bhd2 and Bsn from phage display libraries enriched for phage displaying proteins that bound to carbohydrates (E. Cho, unpublished results). Blast searches (NCBI) indicated that these proteins are similar (38\% and 51\% identity, respectively) to Mpg1 hydrophobin of \textit{Magnaporthe grisea} (E value 9e^{-11}) and the \textit{Neurospora crassa} Snodprot1 toxin (E value 2e^{-35}). Based on the number of sequences between the cysteines, both Bhd1 and Bhd2 are class I hydrophobins (fig. 5-3) (38).

Our previous results (Chapter 3) suggest that \textit{B. bassiana} conidia are hydrophobic, and a rodlet layer is clearly visible by AFM on the surface (Chapter 2). Previous research
(7) of the *B. bassiana* rodlet layer appeared to indicate that it contains a single cell wall hydrophobin (10-14 KDa protein), however we have identified the genes for at least two hydrophobins (*Bhd1* and *Bhd2*) and a hydrophobin like protein (*Bsn*).

Class I:  
\[-C-X_{5.8}-CC-X_{17.39}-CC-X_{8.23}-CC-X_{5.6}-CC-X_{6.18}-C-X_{2.13}\]

H1  
\[
7 \quad 27 \quad 18 \quad 5 \quad 12 \quad 7
\]

H2  
\[
7 \quad 37 \quad 17 \quad 5 \quad 17 \quad 8
\]

Class II:  
\[-C-X_{9.10}-CC-X_{11} -C-X_{16} -C-X_{6.9}-CC-X_{6.18}-C-X_{2.13}\]

Fig. 5-4. Comparison of Bhd1 and Bhd2 and other hydrophobins (A) consensus spacing for Class I and Class II hydrophobins as suggested by Fuchs et al. (38).

At least four major bands were observed when TFA soluble, SDS insoluble proteins were extracted from aerial conidial cell walls (Figure 5-1). Based on reports in the literature discussing *B. bassiana* cell wall proteins that were insoluble in hot SDS, but soluble in TFA or FA hydrophobin (7, 68), we selected the band with a molecular weight of approximately 10-14 KDa (based on SDS page anlaysis) for further analysis because we considered it the best candidate for the cell wall hydrophobin.

Mass peptide spectrometry analysis of the 10-14 KDa band, when compared to the sequence of the two hydrophobins and the hydrophobin like protein, identified the protein as Bhd2. These preliminary results indicate that Bhd2 is a component of the *B. bassiana* cell wall. Aside from aerial conidia *B. bassiana* produces the other two single cell propagules, blastospores and submerged conidia. These cells do not appear have a visible rodlet layer and SDS insoluble, TFA soluble proteins were not isolated from purified cultures of these cells. However, real-time RT-PCR on RNA isolated from these cells indicated that transcripts corresponding to *bhd1* and *bhd2* could be detected in pure cultures of these cell types, although levels of *bdh2* were low in all cell types.

It is likely that conidia are not metabolically active; as reflected by the relatively low levels of tubulin amplified by real-time RT-PCR (Table 5-2). Alternatively, the low
levels of tubulin could be explained by low expression of tubulin alone, with other genes being expressed at relatively higher levels. Using other housekeeping genes, or purifying and quantifying poly A RNA will determine which of these explanations is the most likely.

There are a number of possibilities explaining the low levels of bhd2 RNA observed in the cell types analyzed. It is possible that bhd2 is only produced in sporulating cultures, specifically in cells responsible for producing conidia (conidiophores) and was therefore not expressed by any of the cell types tested. In contrast bhd1 was present in almost all the cultures tested at a relatively high abundance. This protein while not being recruited to the single cell propagule cell walls may instead be secreted into the medium. Similar to other hydrophobins which are not recruited to conidial cell walls it may function as a surfactant, coat surfaces acting as a lubricant, or function as an adhesin directing hyphal attachment and growth.

Bsn, like bhd2, is likely produced under specific conditions not examined in these studies. Based on homology to proteins produced by Pisolithus microcarpus and Ceratocystis fimbriata. Homologous proteins are produced by these organisms are expressed by vegetative mycelium, when the expression levels of other hydrophobins or carbon availability is low. Putative functions for the product of this gene may include host cell toxicity (28, 69).
CHAPTER 6
GENERAL DISCUSSION

Statement of Hypotheses

The work in this provides scientific data to examining the validity of the following hypotheses: 1) that there is a measurable difference in the cell surface characteristics of the single cell propagules of *Beauveria bassiana*, and 2) there are differences in the binding properties of these propagules which can be related to the cell surface characteristics.

Is There a Measurable Difference in The Cell Surface Characteristics of The Single Cell Propagules of *B. bassiana*?

Visual Differences

Visual inspection of the single cell propagules using light or atomic force microscopy reveals that the morphology and the surfaces of *B. bassiana* aerial conidia, submerged conidia and blastospores are different. Aerial conidia are spherical, 2-4 μm in diameter, hyaline and covered with a distinct rodlet layer. Submerged conidia are similar in shape to the aerial conidia but are larger (3-5 μm in diameter), and while the surface appears rough, there was no visible evidence of a rodlet layer in those cells. Blastospores are rod shaped and larger than the other spores at 8-12 μm in length, there is no visible rodlet layer on their surface, which is smooth (35, 83).

Differences in Hydrophobicity

Interfacial free energies of interaction derived from contact angle data and percent retention of the spores in the hydrocarbon phase (MATH) assay, both confirmed that the
conidia have negative interfacial free energies of interaction indicating that they would be unable to overcome the high cohesive force of water, and with almost 88 % retention in the hydrocarbon layer, it is clear that these spores are highly hydrophobic. Submerged conidia were less moderately hydrophilic, with slightly positive interfacial free energies of interaction and 74 % retention in the hydrocarbon layer. Blastospores with strongly negative interfacial free energies of interaction and low retention (40%) in the hydrocarbon layers were the most hydrophilic.

**Differences in Effective Surface Charge**

Effective surface charge (zeta potential) of the three spores was similar in sign (positive at low pH and negative at high pH) as pH changes from 3-9. All of the spores had the same isoelectric point (pH 4), except for conidia that were 16-days-old, the isoelectric point for these cells was pH 5. Conidia registered the greatest magnitude of charge of the three spore types, (zeta potential: -29 mV, day 16 and – 47 mV, day 20) at very high pH (pH 8) and at low pH (pH 3) for 16-day-old aerial conidia (zeta potential: 22 mV. At low pH (ph 3) the zeta potential for all the spores, except for the 16-day-old conidia, was between 3-8 mV. The magnitude of charge for submerged conidia at high pH was intermediate with a zeta potential of -10 mV, and that for the blastospores was the lowest with a zeta potential of – 5 mV. The overall change in zeta potential with respect to pH for the blastospores was 7 mV, while that for the submerged conidia was – 20 mV, and that of the aerial conidia (16 and 20 days combined) was 69 mV.

The conidia that were the most hydrophobic had the highest charge, this could be indicative that: 1) electrostatic repulsion is less of a factor for the more hydrophobic cells because hydrophobic forces are stronger and act over a longer range, 2) that the surface to which aerial conidia bind to tend to be less electronegative at higher pH than aerial
conidia (this is true for insect cuticle; many of the proteins in the insect cuticle tend to be positively charged at higher pH), whereas the surfaces that the blastospores and microccyle conidia bind to tend to be less more at higher pH than either of those cell types, or that 3) aerial conidia are designed to adhere to hydrophobic surfaces, using the surface of water as a dispersion mechanism, whereas blastospores and submerged conidia are designed to be resuspended in aqueous solutions for longer periods of time.

**Cell Wall Proteins**

Sodium dodecyl sulfate (SDS) insoluble, trifluoroacetic acid (TFA) soluble proteins were extracted from the cell wall of conidia, but not from the cell walls of microcycleconidia, these proteins often belong to a class of small, cysteine rich, secreted fungal proteins called hydrophobins (99). The genes for two hydrophobins and one hydrophobin like protein specific to *B. bassiana* have been identified, cloned and sequenced in our laboratory. The *bhd1* sequence was identified from gene fragments obtained from a SSH library designed to find virulence factors upregulated when the organism is grown in the presence of chitin, or insect cuticle. The other two genes were identified by E. Cho in this laboratory from phage display libraries designed to enrich for carbohydrate binding proteins. It has been noted, but never conclusively proven that hydrophobins poses lectin like qualities hydrophobin (93, 99).

The amino acid sequences of the two hydrohobins (Bhd1 and Bhd2) were compared to the sequences of fragments obtained by running peptide fingerprinting on a 12 KDa SDS insoluble, TFA soluble protein isolated from the cell wall of the conidia. The sequence of two of the fragments matched at least 29% of the sequence of Bhd2. To determine the expression patterns of all three genes, real-time reverse transcriptase PCR (real time RT-PCR), was performed on the three single cell propagules and vegetative
mycelia from cells grown on chitin or in sabauourd dextrose broth (SDY) (1-2% YE). 

*bhd1* was expressed in all cell types, *bhd2* was expressed mostly in cells exposed to chitin, and submerged conidia and *bsn* was not expressed in any of the cell types tested.

**Conclusions**

From these results we can conclude that there are measurable differences in the surface characteristics of the three cell types with regards to morphology, surface charge, hydrophobic characteristics, and cell wall protein composition.

**Are there Differences in the Binding Properties of These Propagules, Which Can Be Related to the Cell Surface Characteristics?**

**Adhesion Profiles of Aerial Conidia, Submerged conidia and Blastospores**

Our fluorescent cell, microtitre plate adhesion assay determined that *B. bassiana* aerial conidia bound predominantly to hydrophobic surfaces (silinized polystyrene), and weakly to hydrophilic surfaces (polystyrene, factory-treated to be more hydrophilic). Submerged conidia bound well to either surface, while blastospores bound predominantly to the more hydrophilic surfaces. This correlates well with the hydrophobic character of each spore type, however treating with amylase, laminarinase and protease could reduce the binding of aerial conidia to hydrophobic surfaces, and treatment with maltose reduced blastospores adhesion to hydrophilic surfaces, indicating that factors other than hydrophobicity are implicated in spore adhesion.

**Conclusion**

The surface characteristics of *B. bassiana* single cell propagules are consistent with the adhesion profiles on hydrophilic, and hydrophobic surfaces. However, it is clear that other the adhesion properties of the spores are a result of the interactions of short and long range intermolecular forces. These intermolecular forces are consistent with the
distinct cell wall characteristics of the three cell types, however it is probable that other features not yet identified are contributing to the adhesion characteristics of the cell types.

Confirmation of the role of the Bhd2 hydrophobin in the adhesion profile of *B. bassiana* conidia and the function of the other two *B. bassiana* specific proteins (Bhd1 and Bsn) will require generating transformants lacking Bhd2. The *Agrobacterium tumefaciens* mediated transformation procedure, with minor modifications, will be used for this purpose.

Finally it should be noted that the adhesion profiles and the cell surface characteristics unique to *B. bassiana* must be important in the ability of the fungus to successfully function as an entomopathogen. Although the different propagules have different surface characteristics and adhesion profiles, all are able to successfully initiate infection, the main observable difference between the cells, with respect to use in mycoinsecticidal preparations, is the resistance of aerial conidia to environmental stresses such as UV light and desiccation spores, this difference has previously been correlated to the presence of the rodlet layer on the surface of the aerial conidia (80).
APPENDIX
AGROBACTERIUM MEDIATED TRANSFORMATION OF BEAUVERIA
BASSIANA

Introduction

Little known concerning the regulation of molecules and metabolic pathways involved in fungal pathogen-host interactions particularly for the entomopathogen. This is primarily due to the lack of efficient transformation systems for filamentous fungi. Development of efficient transformation systems for filamentous fungi have been limited to several species particularly those related to human health. Successful genetic manipulation of fungi, as with any organism, requires a plasmid mediated transformation system involving the entry and replication, or integration of foreign DNA. Once DNA has been successfully introduced into the fungal cells its fate varies depending on the host. DNA usually integrates into the chromosomes; autonomously replicating plasmids are rare in most filamentous fungi.

There are a number of transformation strategies currently being used with mixed degrees of success. The earliest, and still widely used, transformation method for filamentous fungi is polyethylene glycol (PEG) mediated transformation of fungal protoplasts. More recently developed methods sometimes used in conjunction with the generation of protoplasts; include lithium acetate treatment, Agrobacterium tumefaciens mediated transformation (ATMT), electroporation, restriction enzyme mediated transformation (REMI), and biolistic transformation. Many of these methods,
simplify transformation procedures, reduce variability, and/or increase transformation efficiencies.

Agrobacterium tumefaciens mediated transformation (ATMT) is routinely used for the genetic modification, primarily of dicotyledon plant species. Agrobacterium tumefaciens causes crown gall in plants by inducing tumor formation, and the over-production of specific nutrients (opines) within the host cell. This is accomplished via the transfer of part of its Ti plasmid DNA to the host genome. The transferred DNA (T-DNA) contains genes encoding auxins, cytokinins and opines; the remainder of the plasmid, and part of the bacterial genome, codes for proteins involved in the detection of acetosyringone, a substance released by wounded plants. Excision, transfer and integration of the T-DNA, into the host genome are not dependent on DNA present in the T-DNA region, which can be artificially modified without affecting the transformation process.

In 1998, de Groot and Gouka demonstrated that efficient transfer of T-DNA to filamentous fungi, specifically to the fungus Aspergillus awamori, was possible, and that transformation frequencies were increased up to 600 fold compared to traditional methodologies (18, 41). Transformation by the same group of other fungi, including Aspergillus niger, Fusarium venetatum and Trichoderma reesei, Colletotrichum gloeosporioides, Neurospora crassa and Agaricus bisporus (a mushroom), demonstrated that this system that could be adapted for a variety of filamentous fungi (18).

Materials and Methods

Fungal Cultures

Beauveria bassiana (ATCC 90517) was routinely grown on potato dextrose agar (PDA) (4). Plates were incubated at 26°C for 10-14 days, and aerial conidia were
harvested by flooding the plate with sterile dH₂O. Conidial suspensions were filtered through a single layer of Mira-cloth (Clabiochem, CA), and final spore concentrations were determined by direct count using a hemocytometer.

Blastospores were produced in Difco™ Sabouraud dextrose (Becton, Dickinson and Co., MD) + 1-2% yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of 0.5 - 5 x 10⁵ conidia/ml as the inoculum. Cultures were grown for 3-4 days at 26°C with aeration (150-200 rpm). Cultures were filtered (2x) through glass wool to remove mycelia, and the concentration of blastospores was determined by direct count.

For all cell types, Mira-cloth (Clabiochem, CA) or glass wool filtered cell suspensions were harvested by centrifugation (10,000 x g, 15 min, 4°C), washed two times with sterile dH₂O, and resuspended to the desired concentration as indicated (typically 10⁷-10⁸ cells/ml).

**Agrobacterium tumefaciens Cultivation**

The transformation was performed as described by Covert et al. (2001) with the following modifications. Conidia (10⁴-7 cfu/ml) were mixed in equal amounts with an overnight Luria Bertani (LB, Difco, MI) broth (40 µg/ml ampicillin and 50 µg/ml kanamycin) culture of *Agrobacterium tumefaciens* (AGL1). The *A. tumefaciens* culture was in diluted to O.D. 0.15 with induction medium (16) supplemented with acetosyringone (IMAS) and allowed to grow for 4 hrs to reach an O.D. of 0.6-0.8.

**Transformation Procedure**

Then 50-200 µl of the *Agrobacterium* and *B. bassiana* cultures were mixed and plated onto sterile cellophane overlaid onto IMAS plates. The plates were incubated for 3 days at 20°C and the filters were transferred to the Czapek-dox (CZD) (Difco, MI)
plates supplemented with the appropriate antibiotic (1000 µg/ml hygromycin B with 350 µg/ml of cefotaxime, or 750 µg/ml G418 with 350 µg/ml cefotaxime).

The fresh plates were incubated at 20°C for 4 to 10 days until isolated colonies were visible. Sensitivity to the various antibiotics tested, was affected by the salt concentration of the medium as well as the pH. It was determined that LB and the M-100 media did not have sufficient buffering capacity. CZD agar gave the best results for sensitivity for all antibiotics tested, and buffering capacity (results not shown).

Putative transformants were restreaked onto selective media and then inoculated into SDY (1-2% YE) broth. PCR reactions were run on purified genomic DNA (GenElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich, MO) of the putative transformants, with primers designed to amplify genes (e.g. hph) present in the T-DNA, on the wild type (WT) chromosome (aspfII).

**Results**

*Agrobacterium tumefaciens-Mediated Transformation*

ATMT with pPK2 resulted in at least 526 colonies able to grow on the selective media, however the background of wild type colonies (growth of colonies on control plates w/o acetosyringone) was sometimes very high. Colony PCR of the initial transformants obtained using pPK2 showed that many of the colonies in the presence of *Agrobacterium* (at least 66%) contained the hph gene (Fig. A-1), which was not amplified from any of the control colonies.
Fig. A-1. PCR analysis of putative *Beauveria bassiana* transformants: A) lanes 4, 7, 10, 13, and 16 positive control for *Beauveria* (AspfII), lanes 3, 6, 9, 12, and 15 amplification of the *hph* gene B) lanes 3, 5, 8, 11, 14, 17 and 20 positive control for *Beauveria* (AspfII), lanes 4, 6, 9, 12, 16, 18, and 19 amplification of the *hph* gene

Transformation with pPk2 derived plasmids containing the neomycin phosphotransferase gene (*neo'*) or *neo'* combined with the gene for green fluorescent protein *gfp* produced hundreds (715) of putative transformants. Many of these (198/717) failed to grow when subcultured to fresh selective media unless the concentration of the antibiotic was reduced in half and none were confirmed to have the *neo'* gene (Table A-1).

ATMT with our strain of *B. bassiana*, using *hph* as the marker, on average produced more colonies (including many background colonies) from blastospores than using *neo'* (≥ 250 colonies/10^3 cells vs. 83.2 ± 24.8 colonies/10^3 cells n=4 plates) (Table A-1). Azaserine, a purine synthesis inhibitor slightly increased the number of putative transformants (*gfp-neo*) from an average of 16 ± 4.24 colonies/10^2 cells to 32.5± 17.6 colonies/10^2 cells (n=2 plates). Blastospores, regardless of selectivity gene, produced
more putative transformants (≥ 250 colonies/ 10^4) on average, than conidia (e.g. 87.7 ± 90.81 colonies/ 10^4 cells) (Table A-1).

Table A-1. Putative transformants obtained from *Agrobacterium tumefaciens* mediated transformation of *B. bassiana* with selection markers for hygromycin B (*hph*) and neomycin (*neo*) resistance.

<table>
<thead>
<tr>
<th>Resistance marker</th>
<th>Conidia 10^3 a</th>
<th>Conidia 10^4</th>
<th>Conidia 10^5</th>
<th>Blastospores 10^2</th>
<th>Blastospores 10^3</th>
<th>Blastospores 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hph</em></td>
<td>-</td>
<td>8^d, 10</td>
<td>-</td>
<td>-</td>
<td>TNTC, TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>200,200</td>
<td>-</td>
<td>-</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>28, 80</td>
<td>-</td>
<td>-</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td><em>Neo</em></td>
<td>-</td>
<td>0^e</td>
<td>0</td>
<td>19</td>
<td>90^f, 74^f</td>
<td>TNTC</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>96, 102</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>37^g, 100^g</td>
<td>-</td>
</tr>
<tr>
<td><em>gfp-Neo</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13, 19</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20^g, 45^g</td>
<td>TNTC^f</td>
<td>-</td>
</tr>
</tbody>
</table>

a. Conidia were obtained from 4-7 day old plates, blastospores were obtained by growing Bb in SAB dextrose broth with 2% yeast extract for 3 days.
b. *Beauveria bassiana* strain 90517 was transformed with plasmids containing the hph gene, Neo’ and gfp-Neo’ markers.
c. Not done
d. Replicate 1, replicate 2 TNTC = to numerous to count
e. No colonies were recovered from plates using conidia and the neomycin resistance marker
f. Unable to unable to subculture colonies on media with 750µg/ml of G418, only on media with 350µg/ml of G418.
g. *Beauveria* was grown with Azaserine added to the media. All other experiments either had TNTC beauveria colonies on all plates, or Agrobacterium over growth

**Discussion**

An *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol was developed for use with *B. bassiana*. This procedure will eventually be used to conduct targeted mutagenesis of the hydrophobins (*bhd1* and *bhd2*), the hydrophobin like protein (*bsn*), and other genes identified in the suppression subtraction hybridization library of genes upregulated in the presence of insect cuticle.

Covert *et al.* (2001) described a method for the ATMT of *Fusarium circinatum*. The protocol and plasmid (pPK2) used in those experiments were obtained from Dr. S. Covert at the University of Georgia. The *A. tumefaciens* strain AGL1, derived from EHA101, was obtained from the American type culture collection (ATTC). The ATMT
procedure described in Covert et al. (2001) was used with B. bassiana with the following modifications: 1) the selective medium was changed from M100 to Czapek-dox, 2) the selective marker in pPK2 is the hygromycin phosphotransferase gene (hph). Eventually, due to B. bassiana’s resistance to hygromycin at levels of 1000 µg/ml, other selective markers were required, and the appropriate plasmids containing genes conferring resistance to Genticin (G418), and Zeocin were obtained, and the time of co-cultivation was increased from 2 days to 3 days.

Other researchers have adapted ATMT for use with B. bassiana The B. bassiana strain used by Fang et al. (2003) was also highly resistant to hygromycin (up to 2 mg/ml). This resistance appears to be strain dependent however; dos Reis et al. (2004) found their strain to be sensitive to concentrations of hygromycin as low as 600 µg/ml transformation (24, 32).

Although we successfully obtained putative transformants using the hph gene, we were unable to prevent overgrowth of background colonies on control co-cultivation plates without A. tumefaciens or, plates without acetosyringone, even at high concentrations (100 µg/ml) of hygromycin B. Changing the selective marker from hph to neo’ increased the number of putative transformants, and reduced the number of background colonies. However, these putative transformants failed to retain resistance to the selective marker when subcultured, although colony PCR indicated that the neo’ gene was present in most of these colonies.

Using blastospores during the co-cultivation yielded more transformants than using conidia and this is probably due to the faster germination rate (Keyhani, unpublished results) of the blastospores compared to conidia. Roberts et al. (2003) reported that
deleting *Saccharomyces cerevisiae* genes involved made the yeast supersensitive to transformation with *Agrobacterium tumefaciens*. They noted that the magnitude of this sensitivity was large, up to three orders of magnitude. The same group showed that plant cells were also supersensitive to transformation when treated with purine inhibitors, including azaserine (74). We used azaserine in our experiments but did not note the same supersensitivity. There was a modest increase in the number of putative transformants obtained in the presence of azaserine, but that increase was not three orders of magnitude larger.

This technique will be used for targeted mutagenesis of the hydrophobins, and other genes that were identified using the subtractive suppression hybridization procedure to find genes that were potentially upregulated when the fungus was growing on insect cuticle.


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BIOGRAPHICAL SKETCH

Diane’s first school was a small British school in Rome, Italy called the Junior English School of Rome. The school only accommodated students up to Form 1 (age 10) so I transferred to a second school; St. Georges English school also located in Rome. After completing her O and A levels in 1986, she applied to the biology program at the University of Maryland at College park (UMCP). By 1991 she had completed her bachelor’s degree in Biology, and she continued on to complete a Master’s in Microbiology at UMCP (presently the department of molecular and cell biology) under the guidance of Dr. S. Joseph. During this period she was involved in a project USDA funded project to develop a detection system for \textit{Salmonella} sp. in meat products. She obtained her Master’s degree in 1995, and continued to work at the same department for a year as a faculty assistant. She subsequently worked as a supervisor for the quality control laboratory at Allen’s Family Foods. In 2001 after having successfully petitioned for a national interest waiver based on her Master’s work allowing her to self sponsor her permanent immigration application, she decided to continue her education and applied to the doctoral program at Florida Institute of technology, in Melbourne Fl, after one year she transferred to the doctoral program in Microbiology and Cell Science at the University of Florida at Gainesville, Florida where she has been working on the analysis of cell surface properties of the entomopathogenic fungus Beauveria bassiana under the guidance of Dr. N. O. Keyhani.