STUDIES ON THE ENTOMOPATHOGENIC FUNGUS *Beauveria bassiana*: MOLECULAR AND IMMUNOLOGICAL CHARACTERIZATION OF ALLERGENS

By

GREG S. WESTWOOD

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2006

To my wife and children; they are all that truly matter.

ACKNOWLEDGMENTS

I would like to extend my deepest appreciation to my mentor, Dr. Nemat O. Keyhani, whose guidance and direction is reflected in every passage of this work. It was under his tutelage that I was able to see my true potential, and understanding of the role research will always play in my life. I would also like to thank the other members of my committee, Dr. Samuel Farrah, Dr. Peter Kima, Dr. Howard Johnson, and Dr. Jeffrey Rollins, for all of the advice and encouragement they extended in this challenging stage of my education.

I owe a debt of gratitude to Dr. Shih-Wen Huang, who provided the patient sera that played a pinnacle role in our study of allergenicity; without his help, this research would not have been possible. In addition, special thanks go out to all individuals, who devoted their time to assistant to this research; especially in reference to the injection of foreign extracts into, and/or the donation of, bodily fluids.

Finally, I would sincerely like to thank fellow graduate students Lawrence Flowers and Nicole Leal whose friendship and scientific insight had a dramatic affect on my personal and educational development.

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Amp	ampicillin
˚C	degrees centigrade
Cam	chloramphenicol
cDNA	complementary deoxyribonucleic acid
ddH_2O	distilled deionized water
DDT	dichloro diphenyl trichloroethane
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ECF-A	eosinophil chemotactic factor A
EDTA	ethylenediaminetetra-acetic acid
EST	expressed sequence tag
HCl	hydrochloric acid
Hr	hour
HRP	horseradish peroxidase
IgE	immunoglobulin epsilon
IgG	immunoglobulin gamma
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kDa	kiloDaltons
LB	Luria Bertani media
LDS	lithium dodecyl sulfate

µg	microgram
µL	microliter
mg	milligrams
min	minutes
mL	milliliters
mM	millimolar
MOPS	3-(N-Morpholino)-propanesulfonic acid
NCF-A	neutrophil chemotactic factor A
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PD	potato dextrose
PMSF	Phenylmethylsulfonyl fluoride
PVDF	polyvinylidene-fluoride
SDS	sodium dodecyl sulfate
SSH	suppressive subtractive hybridization
TBS	tris-buffered saline
Tris	tris hydrozymethyl aminomethane
tRNA	transfer ribonucleic acid
x G	gravity

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

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Greg S. Westwood

August 2006

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

Beauveria bassiana is an entomopathogenic fungus currently under development as a biological control agent against a wide rang of arthropod pests. Although *B. bassiana* has been reported to be non-toxic to vertebrates, its potential allergenicity has not been studied. Fungal allergens constitute a significant proportion of the airborne allergens that affect up to 25% of the population of the industrialized world. This dissertation examines the ability of *B. bassiana* to elicit allergic reactions, and describes the immunological and molecular characterization of IgE binding proteins present in this fungus.

Immunoblot analyses of *B. bassiana* proteins probed with pooled and individual human sera revealed IgE reactive antigens, ranging from 12 to >95 kDa. Variation was noted when blots were probed using individual sera, however a 35 kDa protein was the most frequently reactive *B. bassiana* antigen. Immunoblot inhibitions experiments identified the presence of shared epitopes between *B. bassiana* and the extracts of several common allergenic fungi (cross-reactivity). IgE binding of the 35 kDa protein was not

inhibited by any of the fungal extract tested, indicating the possible presence of a *B. bassiana* specific antigen. Intradermal skin testing confirmed the *in vitro* results, demonstrating allergenic reactions in a number of individuals, including those who have had occupational exposure to *B. bassiana*.

Screening of a *B. bassiana* cDNA library revealed a number of proteins with sequence similarity to major fungal allergens. Full length clones of the *B. bassiana* genes were obtained by 3' and 5' RACE PCR, and designated as; *bbeno1*, *bbf2*, *bbald*, and *bbhex*. All four proteins were expressed in *E. coli*. BbEno1, designated an enolase by sequence similarity, was compared to 20 other fungal enolases including five known to be allergenic and cross-reactive. Phylogenic comparison showed allergenic (and cross-reactive) enolases are not limited to closely related taxa, but are equally distributed throughout the phylogram. Immunoblot analysis of the four *B. bassiana* proteins revealed BbEno1 and BbAld to be reactive to sera IgEs, and therefore represent the first allergens to be identified from the entomopathogenic fungus *Beauveria bassiana*.

CHAPTER 1
INTRODUCTION

History of Allergy

The term allergy was coined in 1906 by a Viennese pediatrician named Clemens von Pirquet to describe a hypersensitive immune reaction in response to a substance other than a typical disease causing agent (Wagner, 1968). The word “allergy,” was derived from the Greek words *allos* meaning "other" and *ergon* meaning "reaction" or "reactivity."

The word allergy is most commonly used in reference to type I, or immediate onset, hypersensitivity which is characterized as an inflammatory reaction caused by excessive activation of IgE bound mast cells in response to a specific but typically benign antigen. The most common clinical allergy symptoms, hay fever, include runny nose, itchy eyes, and sneezing; however severe allergic reactions can lead to anaphylactic shock and even death (Gould et al., 2003; Kurup and Banerjee, 2000).

Allergens known to affect large groups of people are designated as major allergens and are typically common place in the air we breathe (Table 1-1); a recent survey found that over 54% of US citizens tested positive for sensitivity to at least one allergen (AAAAI, 1996-2001; Arbes et al., 2005). Outdoor allergens include industrial pollutants, pollens, and other plant materials; common indoor allergens include pet dander, dust mites, and cockroach feces. Fungal spores constitute a significant portion of both indoor and outdoor major allergens.

Type I hypersensitivity is a growing problem; allergic disease is projected to effect 20–25% of the population of the world's industrialized nations and of those, 10% develop severe allergic disease (Horner et al., 1995; Kurup et al., 2002). The National Institute of Allergy and Infectious Diseases estimate that about 50 million Americans are affected by allergic diseases in the United States alone, with allergies constituting the sixth leading cause of chronic disease. The cost associated with allergic disease is estimated to exceed 18 billion dollars annually (AAAAI, 1996-2001; Sagi-Eisenberg, 2002).

Hypersensitivity

Hypersensitivity results from over stimulation of the immune system to an antigen, considered benign typically. Hypersensitivity has been characterized immunologically into four types based on their clinical symptoms and underlining mechanism (Horner et al., 1995).

Type I, or immediate, hypersensitivity is an activation of mast cells and basophiles by antigen specific membrane bound IgEs. Type II is mediated by the binding of IgE or IgM to a specific antigen on the surface of a cell leading to the destruction of the cell. This process often involves the classic complement pathway and is usually associated with hemolytic disease. Blood group incompatibility is an example of type II hypersensitivity. Type III is caused by the formation of antigen antibody (ag-ab) complexes of circulating IgGs, which bind to and activate mast cell via FcγRIII low affinity receptors. Ag-Ab complexes also interact with blood vessel walls which are damaged by a massive infiltration and degranulation of neutrophils activated by the mast cell cytokines. Type IV or delayed hypersensitivity is mediated by antigen specific Th1 cells, which lead to the release of cytokines responsible for the recruitment and activation of T-cells and macrophages.

Traditionally the term "allergy" is used to refer to conditions caused by type I (immediate) hypersensitivity; however, many allergic or hypersensitive disease are not cause by a single type, but result from the combined effects of two or more types. This has led to a broader definition of the term allergy. The Institute of Medicine defines allergy as "The state of immune hypersensitivity that results from exposure to an allergen and is distinguished by overproduction of immune system components" (Pope, 1993). In this dissertation the term allergy is used to describe diseases or conditions that are caused by type I directly, or in which type I plays an essential role.

Immediate Hypersensitivity

A type I hypersensitive or allergic reaction is mediated by antigen specific IgEs bound to mast cell and basophiles by a high affinity surface receptor, FcεRIb (von Bubnoff et al., 2003). The reaction is initiated when the binding of an allergen leads to the cross linking of two receptor bound IgEs (Figure 1-1). The cross linking of IgEs triggers a host of cellular responses resulting in the release of several chemical mediators (Table 1-2). The first and most dramatic of these is the immediate degranulation of storage vacuoles containing the primary vasoactive amine mediators as well as molecules including proteases, hydrolases, and chemotactic factors (Kawakami and Galli, 2002). These mediators and associated factors are responsible for the clinical symptoms associated with an immediate inflammatory response. The cross linking of FcεRI receptors also initiates the *de novo* synthesis of secondary mediators including leukotrienes and cytokines which are responsible for the onset of the late-phase inflammatory response (Sagi-Eisenberg, 2002).

The vasoactive amine, histamine, is the dominant molecule released by the initial degranulation of an allergen-activated mast cell (Shim et al., 2003). It is responsible for

triggering numerous cellular responses depending upon the nature of the surrounding tissue. Its primary function, however, is as a vasodilator leading to vessel leakage and swelling, or inflammation of the surrounding tissue. Proteases including, chimase and tryptase exacerbate this process by degradation of blood vessels and basement membrane. Chemotactic factors including ECF-A (eosinophil chemotactic factor A) and NCF-A (neutrophil chemotactic factor A) lead to an influx of secondary leukocyte. Activated by the mast cell mediators these secondary leukocytes secrete their own mediator molecules causing additional tissue damage as well as the recruitment of even more leukocytes.

Leukotrienes and prostaglandins are arachidonic acid metabolites that act as secondary mediators. They increase vessel permeability and cause contraction of pulmonary smooth muscles. Other cytokines produced by mast cells act in the recruitment and activation of platelets and leukocytes, drawn into the area by the chemotactic factors. The actions of leukocytes, such as eosinophils and neutrophils, result in the clinical symptoms of the late phase reaction (Goldsby, 2000).

Allergic Disease

Atopic allergic disease refers to the immediate hypersensitive response mediated by IgE. Allergic response occurs at the location of antigen contact, and the most common tissues affected are those of the respiratory and digestive tracks, although the eyes and skin are also susceptible to contact with allergens.

Allergy to aeroallergens or hay fever is one of the most common allergic diseases and is characterized by symptoms including rhinitis, coughing, sneezing, nasal discharge, and conjunctivitis (itchy or watery eyes). More severe cases can lead to constriction of bronchia (asthma) manifested as a shortness of breath (Shim et al., 2003). Skin reactions

to dermal contact with an allergen include urticaria and eczema whose symptoms include swelling and itching.

Potentially deadly reactions to allergens are usually associated with food allergies and insect venom. The response to an ingested allergen can manifested as abdominal pain, vomiting, diarrhea, and/or swelling of the tongue and lining of the throat. In severe cases, the swelling can lead to a complete closing of the airway. Anaphylaxis is an acute systemic response to an allergen in the blood stream. The release of histamine by blood basophils and mast cells into the circulatory system leads to vessel leakages causing swelling, itching, and hives. Constriction of pulmonary smooth muscles leads to difficulty breathing. Anaphylactic shock is a potentially life threatening form of anaphylaxis in which systemic degranulation of mast cells and blood basophils, leading to constriction of airways, a rapid loss of blood pressure, and shock . This is most often associated with an allergen entering the blood stream by ingestion or injection (insect venom and pharmaceuticals).

Type I hypersensitivity also plays a fundamental role in chronic allergic disease. Chronic allergic disease is usually caused by a combination of hypersensitive types including type I. The most common is allergic asthma, which is a form of localized anaphylaxis. Degranulation of mast cells in the lungs causes excess mucus secretion, airway edema, and constriction of pulmonary smooth muscle resulting in airway obstruction (Goldsby, 2000). Seventeen million Americans are afflicted with asthma, which is responsible for more than 5,000 deaths annually (CDC, 2002; O'Hollaren, 2006).

Allergic bronchopulmonary aspergillosis (ABPA) is an inflammatory disease caused by fungal growth in the mucous of the lungs, typically due to infections by *Aspergillus fumigatus* (but can be caused by fungi of other genera) (de Almeida et al., 2006; Denning et al., 2006). Extrinsic allergic alveolitis is a lung disorder resulting from hypersensitivity to inhaled allergens such as fungi and organic dust; this disorder also involves components of the type III and type IV hypersensitivity responses (Bush et al., 2006; Horner et al., 1995).

Fungi

The "Fungi" represent a taxonomic kingdom comprised of both multicellular and single cellular eukaryotic organisms. Fungi display a wide morphological diversity, ranging from large mushrooms to microscopic yeasts. Many fungal species are dimorphic and can persist and grow in either a single or multicellular state depending upon environmental conditions. There are currently over 100,000 recognized species of fungi, distributed throughout almost every ecosystem including Antarctica (Palmer and Friedmann, 1988). The largest and most common group of fungi is the Ascomycetes, which are primarily the filamentous mold fungi, but also include some single celled species.

Spores and Conidia

The fungal life cycle is divided into two stages: sexual and asexual. Many fungi are able to reproduce both sexually and asexually. Fungi capable of reproducing sexually are termed "perfect" and are considered to be in a telemorphic or sexual state. Sexual reproduction results in the creation of sexual spores. When a fungus is reproducing asexually it is considered to be an anamorph and the end result is the production of conidia. Fungi that reproduce exclusively in an asexual state or for which no sexual stage

has yet been identified are classified as “imperfect” or anamorphic fungi. The Deuteromycetes represent a sub-grouping of filamentous fungi within the Ascomycetes that are considered to be strictly anamorphic. Both sexual spores and conidia are propagules released by the parent organism; the term spore is used in this paper to describe both sexual spores and asexual conidia. Spores and conidia are considered relatively more resistant to unfavorable environmental conditions than other cells, and are designed to stay metabolically inactive until environmental conditions are favorable for supporting growth. The availability of water (high humidity) is usually a major factor in the germination of spores and conidia (Cole and Kendrick, 1981; Lacey, 1981).

Because of their size, spores are easily dispersed in the air and are found aerosolized in the atmosphere throughout the world. Aerobiological assessments of indoor and outdoor fungal spores have often been used to determine the identity and concentration of aerospores (Al-Suwaine et al., 1999; Beaumont et al., 1985b; Kurup et al., 2000a). Outdoor concentrations of fungal aerospores often outnumber pollen counts one hundred to one thousand fold (Horner et al., 1995; Lehrer et al., 1983) and are directly affected by climatic events such as, precipitation and wind. Although seasonal variations in fungal aerobiological numbers have been noted, this variation is much less than that observed for pollens. *Alternaria*, *Cladosporium*, *Epicoccum*, and *Fusarium* are examples of outdoor fungi typically associated with human allergy. The fungi that dominate indoor air are those that commonly grow indoors, and include species of *Aspergillus* and *Penicillium*. The indoor concentration and type of fungal aerospores is more dependent upon carpet, houseplant, and humidity conditions than outdoor seasonal or climate changes (Kozak, 1979; Salo et al., 2005). Outdoor fungi can also be found

indoors and their concentrations are affected by factors that facilitate entry such as traffic, pets, and ventilation.

Health

As with all fungi, filamentous fungi acquire nutrients by absorption and are generally saprophytic or symbiotic; although there are some fungal species that are parasitic and/or opportunistic pathogens. In recent years, fungi have become an ever-increasing health concern. Immunocompromised patients, particularly those with AIDS, are highly susceptible to sometimes fatal infections by opportunistic fungi. This is also true for transplant patients in which the immune system is suppressed to avoid organ rejection. Chemo- and radio-therapies for cancer treatment also weaken the immune system, increasing the risk of cancer patients to infection by opportunistic fungi. With the increasing population of individuals with compromised immune systems, there is also an increase in infection by opportunistic fungi such as *Aspergillus fumigatus* and *Histoplasma capsulatum*.

In otherwise immune competent individuals, fungal allergies are another serious health concern. Atopic allergy affects up to 25% of the population of industrialized nation with clinical symptoms ranging from sneezing and coughing to chronic sinusitis and asthma. Allergens affect the area that they come in contact with which includes the skin and mucosal layers of the nasal and respiratory tract. For inhaled particles the size of an aeroallergen will determine the location in the respiratory track that the allergen will interact with host tissues initiating a response. Large particles (>10 μm) such as dust, large spores, and pollen cause upper respiratory problems primarily in the sinuses and nasopharynx (Lieutier-Colas et al., 2003). Smaller particles (<5 μm) including many fungal spores penetrate deeper in the respiratory track often affecting the bronchia and

lungs resulting in asthma (Nygaard et al., 2004; Spieksma, 1995). Fungi are responsible for both upper and lower allergy symptoms. Over 80 genera of fungi have been associated with respiratory track allergy symptoms (Edmondson et al., 2005; Nierman et al., 2005; Schwienbacher et al., 2005) and 25–30% of all allergic asthmas cases have been linked to mold allergies(Kurup et al., 2002; Vijay and Kurup, 2004). The viability of a fungal spore is not required for eliciting allergenic reactions; nonviable spores and hyphal particles are still able to cause allergic reactions.

Nomenclature

As allergy research identifies an ever-increasing number of allergens, a new method for naming allergens was set forth by the International Union of Immunologic Societies Subcommittee for Allergen Nomenclature (Marsh, 1987). Before definitive naming of an allergen, several criteria have to be met; (1) the allergen must be identified by multiple immunochemical or physiochemical techniques, and (2) the source of the molecule must be clearly defined including species and strain in the case of fungi. An allergen is labeled by the first three letters of the genus followed by a space and the first letter of the species and then a space followed by a number assigned based upon the chronologic order in which the allergen was identified. The strain number is the final addition to the new name. In reference to the gene producing the allergen the Arabic numerals are substituted by Roman numerals (King et al., 1994; King et al., 1995). Inconsistency in the assigned number of an identified allergen can be observed between the publications of different research group. In this dissertation, the allergens are designated by the name used by the International Union of Immunological Societies Allergen Nomenclature Sub-committee List of Allergens (Milligen, 2006).

Major Allergenic Fungi

Skin test and aerial surveys have identified the most significant fungi associated with human allergy. Some of the most common genera are: *Alternaria*, *Cladosporium*, *Epicoccum*, *Aspergillus* and *Penicillium* (Cruz et al., 1997; Kurup and Banerjee, 2000; Shen and Han, 1998); all of which belong to the group Ascomycota.

Alternaria alternata

Alternaria alternata is one of the most important allergenic fungi (Herrera-Mozo et al., 2006; Kurup et al., 2003; Salo et al., 2006). *A. alternata* is a dematiaceous mold found in the soil, on plants, and in the air throughout the world preferring climates that are warm and moist (Achatz et al., 1995; Pritchard and Muir, 1987). A member of the Deuteromycetes, *A. alternata* is a health concern as both an opportunistic pathogen and a major allergen (Vartivarian et al., 1993). Although not pathogenic in immune component individuals, *Alternaria* is of concern, because it can cause both atopic and asthmatic reactions. Indeed, allergy to *Alternaria* is considered to be a mortal risk factor in asthma patients (Chiu and Fink, 2002). *Alternaria* allergenicity has been closely examined and a number of allergens have been isolated and characterized from this organism (Table 1-2) (Achatz et al., 1995; Kurup et al., 2002).

Alt a 1 is the most common allergen of *Alternaria alternata*. Although its biological function is not known (Saenz-de-Santamaria et al., 2006), it is a secreted homodimer roughly 60 kDa (monomer mw roughly is 30 kDa). Of patients displaying allergic reactions to *Alternaria alternata*, 80% are positive for IgE reactivity to Alt a 1. One factor that contributes to the extreme allergenicity of Alt a 1 is that it contains four IgE binding region (Kurup et al., 2003). Other allergens identified in this fungus include Alt a 6, an enolase, and Alt a 10, a protein with aldehyde dehydrogenase activity, both of

which are considered common "house-keeping" enzymes. The enolase has been of particular interest in resent years due to its homology to allergenic enolases found in other fungi; Alt a 10 is also shares sequence similarities with a known allergen of *Cladosporium herbarum* (Cla h 3).

Cladosporium herbarum

Cladosporium herbarum is a highly allergenic fungus not only because of the large number of allergens it produces, but also because of its relatively high abundance in the environment. *C. herbarum* is found throughout the world and is often the dominant outdoor airborne fungal spore especially in temperate regions (Achatz et al., 1995; Lacey, 1981). *Cladosporium herbarum* is a dematiaceous filamentous mold which grows primarily on rotting organic material; although one of the most allergenic fungi, it is not considered pathogenic (Sutton, 1998). There are at least 36 identified allergens produced by *C. herbarum* (Aukrust, 1992). Cla h 1 may be considered the most important *Cladosporium* allergen due to its reactive frequency; 61% of *C. herbarum* sensitive patients possess IgEs that react positively to Cla h 1 (Achatz et al., 1995). Many *C. herbarum* allergens display sequence similarity to allergens produced by other fungi, especially those found in *Alternaria*. Cla h 3 is an aldehyde dehydrogenase that is similar to an enzyme allergen found in *A. alternata* (Alt a 10), and Clah h 6 displays similarity to enolases found in several other fungi including *A. alternata*.

Aspergillus

The genus *Aspergillus* contains several important species known to cause health problems in humans (Garrett et al., 1999; Steinbach and Stevens, 2003). *Aspergillus* species are filamentous fungi found ubiquitously throughout the world and are commonly found in the soil and the air both indoors and out. Although teleomorphic states have

been described for some species, many *Aspergillus* species are Deuteromycetes. The incidence of mediated *Aspergillus* infections, that can sometimes be fatal, has dramatically increased with the increase in patients with compromised immune systems. In addition, this mold is the leading cause of ABPA (allergic bronchopulmonary aspergillosis) and is one of the major risk factors leading to allergic asthma (Crameri et al., 1998a; Crameri et al., 1998b).

Numerous *Aspergillus* allergens have been isolated and characterized, many of which show strong similarity to allergenic proteins or enzymes of other fungi. However, a number of allergens appear to be unique to *Aspergillus* with no known biological functions or activities. Asp f 5 is an *Aspergillus* allergen to which many patients are reactive, its function is unknown and there are no know homologs of this protein. Asp f 5 however, is strongly reactive to sera IgEs of ABPA patients as well as asthmatics (Banerjee and Kurup, 2003; Crameri et al., 1998a; de Almeida et al., 2006). Asp f 1 is a ribonuclease, a ribotoxic virulence factor secreted by the opportunistic fungi that reacts positively with 68–83% of patients who have tested positively for *Aspergillus* allergy. Asp f 8 is a P2 ribosomal protein (60s portion of the large ribosomal subunit) that is similar to allergens found in *Cladosporium herbarum* and *Alternaria alternata*. It is unclear how a ribosomal protein elicits such a strong IgE reaction, however many patients display IgEs that are reactive to all three proteins. The same is true for the *Aspergillus* enolase (Asp f 22), in which an IgE sensitized to one protein is reactive to proteins from different fungi (Hemmann et al., 1997). These data imply conserved allergenic “hotspots” in terms of protein structure and recognition of specific epitopes.

Cross-Reactivity

An interesting fact about fungal allergy is that few patients are allergic to a single fungus. One study testing 6,000 allergy patients showed that more than 99% of the patients allergic to *A. alternata* also displayed allergic reactions to other fungi (Horst et al., 1990). Possible factors that may account for this observation include constant exposure to different fungal spores due to seasonal and localized abundance of fungal spores and the aerosolized mechanism of exposure. These factors make it possible for the immune system to come in contact with numerous species of fungi and fungal antigens that favor an IgE response. Another reason for allergic reactions to multiple fungi is due to IgE recognition of fungal antigen epitopes independent of previous exposure.

Cross-reactivity is a common phenomenon among IgEs produced in response to fungal epitopes that result in the induction of an allergic reaction (Aukrust and Borch, 1985). Cross-reactivity is different than parallel allergy, which is the development of multiple IgE responses to multiple, but similar, antigen. Development of allergies to parallel antigens, such as the same enzyme from two different organisms, is caused by the actions or behaviors of the antigens and result in allergic reactions mediated by different IgEs. Cross-reactivity is a single allergic reaction to multiple antigens; this is the result of structural resemblance and recognition by the same specific IgEs to a single epitope found on all of the antigens (Herrera-Mozo et al., 2006). A large contributor to this phenomenon is that many fungal epitopes are highly conservered especially between phylogenetically related species (Horner et al., 1995).

Enolase, an enzyme responsible for the glycolytic conversion of 2-phospho-D-glycerate to phosphoenolpyruvate is an example of a fungal allergen that elicits an IgE-

mediated response known to be cross-reactive with other fungal enolases. Extensive studies of this allergen by Breitenbach and Simmon-Nobbe have shown that specific epitopes are shared by enolases produced by at least five common fungi; this has led it to being referred to as a pan-allergen (Breitenbach et al., 2002; Breitenbach and Simon-Nobbe, 2002). Enolase was first identified as an allergen in *Saccharomyces cerevisiae* and *Candida albicans* (Baldo and Baker, 1988; Ishiguro et al., 1992; Ito et al., 1995). Later studies concentrated on the enolase allergen Cla h 6 and Alt a 5 that are 90% identical, and were highly IgE immunogenic. Twenty-two percent of patients allergic to either *A. alternata* or *C. herbarum* produced IgEs that reacted with the enolase protein. Competitive inhibition experiments have been performed to analyze cross-reactivity of enolases produced by *A. alternata, A. fumigatus, C. herbarum, S. cerevisiae,* and *C. albicans*. The results of these experiments showed that the examined enolases possessed a shared epitope. Although, the enolase produced by *C. albicans* also displayed a second IgE binding epitope (Simon-Nobbe et al., 2000). An enolase produced by *Hevea brasiliensis* (rubber tree) that was than 63% similar to the enolases Alt a 6, and Cla h 6, was also shown to display allergenic cross-reactivity (Breitenbach and Simon-Nobbe, 2002; Posch et al., 1997; Wagner et al., 2000).

The clinical implication of cross-reactivity is that a patient who develops an allergy towards one species of fungus can have that allergy activated by contact with other fungal species, even those to which there had been no previous exposure. Examples of cross-reactive allergens are listed on Table 1-4 as taken from (Breitenbach and Simon-Nobbe, 2002; Kurup and Banerjee, 2000).

Beauveria bassiana

History

In the early 1800s, the silkworm farms of Italy and France were plagued with diseases that periodically decimated the European silk industry. The disease was called white muscardine after the French word for bonbons, as the disease resulted in fluffy white corpses resembling pastries. An Italian scientist named Agostino Bassi discovered that the disease was caused by a microbial infection and that it could be controlled by altering the living conditions of the silkworms to decrease the spread of the disease. One simple recommendation that he made was to remove and destroy infected and dead insects. Later the microbe, a filamentous fungus, responsible for the disease was named *Beauveria bassiana* in honor of Bassi's discovery. In 1835 Agostino Bassi, one of the founding fathers of insect pathology, published his findings in a paper entitled *Del mal Del segno, calcinaccio o moscardino*; this publication was one of the first instance of a microbe identified as the causative agent of an infectious disease (Alexopoulos, 1996).

B. bassiana is considered non-pathogenic to vertebrates; although there are a handful of recorded cases of human infection by this fungus (Kisla et al., 2000; Tucker et al., 2004). These cases however, involved patients with compromised immune systems increasing their susceptibility to a wide range of opportunistic infections. Based upon safety tests and considered a "natural product," *B. bassiana* has been approved by the U.S. Environmental Protection Agency for commercial use. *B. bassiana* is non toxic to mammals, birds, or plants; and use of *Beauveria* is not expected to have deleterious effects on human health or the environment (EPA, 2000). Strains and various formulations of *B. bassiana* are available commercially in various parts of the world (commercial companies include Mycotech corp. and Troy bioscience U.S.).

Physiology/Life Cycle

Beauveria bassiana is considered to be the anamorph of *Cordyceps bassiana*, an ascomycete in the order Clavicipitales. The genus *Cordyceps* and its anamorph *Beauveria* are endoparasitic pathogens of insects and other arthropods (Nikoh and Fukatsu, 2000).

B. bassiana is a polymorphic fungus whose life cycle includes both single and multicellular stages (Figure 1-2). *B. bassiana* is an ubiquitous saprobe and can be found in soil or decaying plant material, where it grows as multicellar mycelia by absorbing nutrients from the decaying matter (St-Germain, 1996). Reproduction and dispersion of progeny is accomplished by the production of asexual spores called conidia. Conidia of *B. bassiana* are smaller than most other fungal spores measuring only 2–4 μm wide (Akbar et al., 2004; Bounechada and Doumandji, 2004). Conidia are produced from conidiogenic cells that protrude in a zigzag structure from mycelia hyphae. Conidia released into the environment remain dormant or in a non-vegetative state until appropriate conditions activate germination. Humidity is a major factor in activation of conidia independent of a host (Boucias et al., 1988). Attachment of the conidia to the exoskeleton of a host insect also stimulates germination. The initial attachment of *B. bassiana* conidia to the host exoskeleton is thought to be a function of hydrophobicity which creates a strong interaction between the conidia surface and the waxy layer/chitonous surface of the host (Holder and Keyhani, 2005). Germination involves the development of a hyphal structure called a germ tube; the germ tube grows along the surface of the cuticle, and can penetrate into the cuticle by enzymatic digestion and mechanical rupture of exoskeletal components. Once through the exoskeleton, the fungus reaches the hemolymph and there in produces single celled morpho-types known

as *in vivo* blastospores. These cells replicate by budding and proliferate within the hemolymph, evading any innate immune responses (Lord et al., 2002). When nutrients in the hemolymph are consumed the blastospores produce elongating hyphae. These hyphae grow until they exit the cadaver and begin producing conidia one the insect surface. The result is a fuzzy white mummified insect corpse.

Agricultural/Economic Importance

Agricultural pests continue to be a major problem, responsible for tremendous losses in productivity. Traditionally, chemical pesticides such as DDT (dichloro-diphenyl-trichloroethane) and endosulfan have been used to kill unwanted insects. The use of chemical pesticides, however, has resulted in numerous problems. Many insects develop resistance to chemical poisons making these compounds less effective, and therefore required in higher concentrations. Furthermore, extensive application of chemicals into the environment often has deleterious effects on non-target organisms including beneficial insects such as pollinators and natural predators of the target pest. Finally, chemical pesticides display significant health risks to workers who are exposed to the chemicals in the fields as well as to consumers who purchase food products with residual pesticides. Thus, there is great interest in alternatives to chemical pesticides.

The use of biological pesticides such as entomopathogenic fungi is growing in popularity because it is able to alleviate many of the concerns associated with chemical poisons. First, entomopathogenic fungi are found ubiquitously in the soil throughout the world, therefore they would not be considered as "introduced" organisms into the environment. Second, although *B. bassiana* is considered a broad-spectrum insect pathogen, strains can be developed that are more hosts specific. With research into

pathogenicity and strain specificity, it is anticipated that fungal biological control agents can be selected to target specific insect pest.

There are extensive efforts to study/develop *Beauveria* as a biological agent. *Beauveria* has been examined as a potential biological control agent of *Ocneridia volxemi*. A species of grasshopper, *O. volxemi* is one of the most destructive pests of cereals crops in Algeria (Bounechada and Doumandji, 2004). *Beauveria* is also being examined as method to control the citrus rust mite, *Phyllocoptruta oleivora*, a citrus crop pest of South America (Alves et al., 2005). One of the most destructive pests being targeted by application of *Beauveria* control is the coffee berry borer (*Hypothenemus hampei*), which is endemic to most coffee growing regions and results in up to 40% losses of the crop.

H. hampei is an agricultural pest responsible for hundreds of millions of dollars in loses by coffee growers each year (Posada et al., 2004). Beauveria is studied around the world as an effective control agent of coffee berry borer including research facilities found in Honduras, Brazil, Mexico and India (Fernández PM, 1985; Haraprasad N, 2001). Due to the illegalization of some pesticides including enosulfan; Columbia is an example of a country that utilizes *Beauveria* against this pest (Cruz et al., 2005).

B. bassiana as well as *Metarhizium anisopliae* are under investigation and show promise for the control of the tobacco spider mite. The tobacco spider mite is one of several species of mites belonging to the genus *Tetranychus*. Found throughout the United States *Tetranychus* mites are responsible for the destruction of crops ranging from fruits and vegetables to cotton and decorative plants. Studies showed that the treatment of mite-infected tomato plants with conidia of these entomopathogens greatly reduced the

number of mites on the treated plants as compared to untreated plants (Wekesa et al., 2005).

Disease Control

As agricultural pests present an economic and resource production problem to human society, other arthropod pests are a direct human health concern. In this regards, a number of parasitic arthropods act as vectors for the transmission of infectious diseases. Because of their ability to access the human circulatory system, blood feeding arthropods, are important vectors by which microbial parasites can be transmitted between various hosts. *B. bassiana* shows potential for controlling arthropod disease vectors, and hence has the potential to decrease the spread of diseases carried by these insects. Ticks are an example of an arthropod that can carry and transmit a wide variety of disease causing agents. Ticks, obligate blood feeders, are potential carriers of the bacteria *Borrelia burgdorferi*, the causative agent of Lyme disease in humans and domestic animals (Stricker et al., 2006). Other tick born diseases include; R*ickettsia rickettsii*, causative agent of Rocky Mountains spotted fever in both humans and some domestic animals; *Babesia canis*, and *B. gibsoni*, a protozoan parasite of domestic animals; and several species of the genus *Ehrlichia*, an obligate intracellular cocci responsible for a variety of blood cell diseases in domestic animals (Ettinger, 2000; Waner T, 2001). Research studies have shown that the prominent tick species including those known to transmit Lyme disease are susceptible to infection by *B. bassiana* (Kirkland et al., 2004).

Chagas disease is a parasite infection that is transmitted by an insect vector, primarily the South American kissing bug (*Triatoma* infestans) (Lazzarini et al., 2006). Chagas disease is a serious health problem in South America where approximately 20 million people are infected. The health costs associated with treating an infection is often

too high for the majority of those inflicted with the disease. For this reason, research into the control and prevention of the disease, is focused on vector control and involving the use of *B. bassiana* and other entomopathogenic fungi. Brazil and Argentina are two countries with research facilities studying the pathogenicity of *Beauveria* toward these insect disease vectors (Luz and Fargues, 1998; Luz et al., 1998; Marti et al., 2005).

B. bassiana may also be a valuable tool in the fight against malaria. Between 300 and 500 million people are infected with malaria, and this disease is responsible for as many 1.5 million deaths annually (Geetha and Balaraman, 1999; O'Hollaren, 2006). Currently there are no vaccines against malaria; however, studies have shown the potential for fungal entomopathogens to reduce the spread of this disease (Blanford et al., 2005; Scholte et al., 2005). In this regard, the use of entomopathogenic fungi resulting in the infection of as little as 23% of the indoor mosquitoes reduced the yearly number of bites received by residents by as much as 75%. Indoor treatment combined with outdoor applications to control mosquito populations at "hot spots" it is projected that bites by mosquitoes could be lowered by as much as 96% (Scholte et al., 2004; Scholte et al., 2005).

Research Overview

Although not considered pathogenic to humans, the potential for *B. bassiana* to elicit allergic reactions has not been studied. *B. bassiana* may pose a certain level of health concern due to immune responses or hypersensitivity to this organism, as has been reported for other filamentous fungi. Although *B. bassiana* has not been extensively studied as a source of allergenic molecules, a study performed in the Netherlands indicated potential effects (Beaumont et al., 1985a; Beaumont et al., 1985c). A volumetric survey of aero-conidia revealed that *B. bassiana* was one of the most

allergenic species examined although its environmental concentration was too low to be considered important. Although in most cases examined, *B. bassiana* does not persist greater than a couple of weeks after application, with the use of organic pest control agents such as *B. bassiana* the environmental concentration of these fungi may increase resulting in short term exposure of individuals working directly with the fungi, such as those involved in the picking and processing of agriculture crops treated with *B. bassiana*; or those who live in homes treated with *B. bassiana* to control nuisance pest like roaches and earwigs.

This dissertation reports the characterization of *B. bassiana* human reactive antigens. Crude extracts of *B. bassiana* were shown by immuno-blot assays to react strongly with human IgE. This was accomplished with the use of human sera from patients displaying allergic reactions to other fungi. The protein nature of these allergens was confirmed by digestion of the antigens with Proteinase K. The allergenicity of *B. bassiana* extracts varied greatly between individual seras. Intradermal skin testing confirmed the *in vitro* results, demonstrating allergenic reactions in a number of individuals, including those who have had occupational exposure to *B. bassiana*.

Furthermore, the cross-reactive nature of *B. bassiana* allergens was examined. Competitive inhibition experiments where performed using extracts of several know allergenic fungi, including *Aspergillus fumigatus, Cladosporium herbarum, Candida albicans, Epicoccum purpurascens,* and *Penicillium notatum.* The treatment of *B. bassiana* extracts with sera pretreated with other fungal extracts (immunoblot inhibition) resulted in the loss of several bands visible in the, untreated sera, control lanes. A strong

band with the approximate molecular mass of 35 kDa was uninhibited by any of the tested extracts, and may represent a *B. bassiana* specific allergen.

Several potential allergens were identified by homology from a *B. bassiana* cDNA library. The full length genomic and cDNA sequences of four putative allergens were isolated. The genes coding for all four where cloned into over-expression vectors and the proteins expressed in *E. coli*. Using sera from 20 patients BbEno1 was found to react with IgEs in more than 50% of the sera tested, expressed BbAld displayed reactivity IgEs from 4 sera pools, whereas no reactions were observed for the *E. coli* expressed BbF2 and BbHex proteins. Phylogenic comparison of *B. bassiana* enolase shows highly conserved sequence characteristic with the glycolytic enolases of *Cladosporium herbarum* and *Alternaria alternata* both highly cross-reactive fungal allergens.

Table 1-1. Common allergens

Animal	Cockroach
	Cat and Dog dander
	Insect venom (bee, wasp, hornet)
	Mouse urine
	Rat urine
	Dust mites
Foods	Eggs
	Milk
	Peanut
	other nuts (almonds, cashews, etc)
	Wheat
	Fish and Shellfish
	Soy
Plants	Ragweed (*Ambrosia artemisiifolia)*
	Bermuda grass (*Cynodon dactylon*)
	Mulberry (*Morus rubra*)
	Sycamore tree (*Plantanus occidentalis*)
	Cottonwood (*Populus deltoides*)
	American elm (*Ulmus americana*)
	Rye (*Lolium perenne*)
Fungi	*Alternaria alternata*
	Cladosporium herbarum
	Aspergillus spp.
	Penicillium chrysogenum
	Epicoccum nigrum

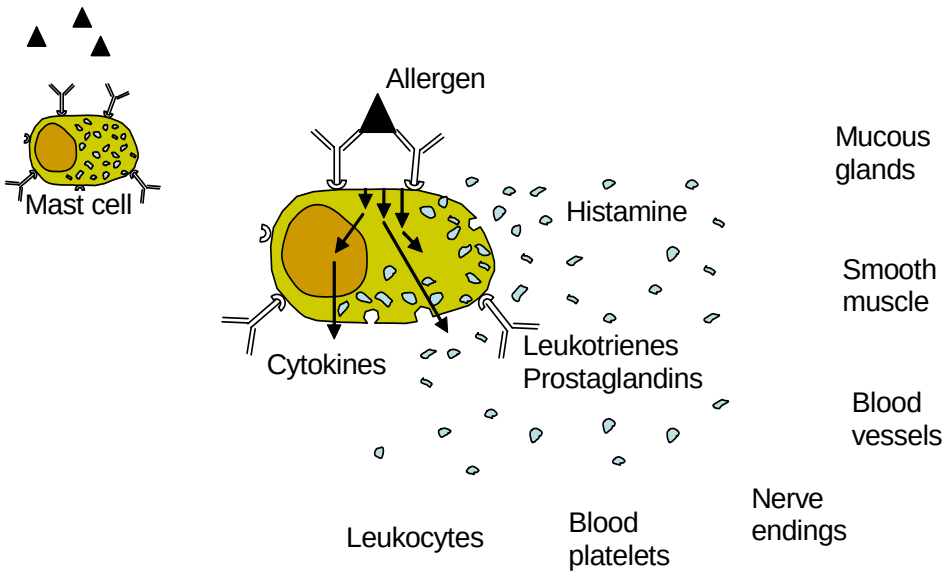

Figure 1-1. Illustrating the central role of IgE activated mast cells in a Type I hypersensitive response; the release of chemical mediators, and the primary tissue types affected by mast cell chemicals.

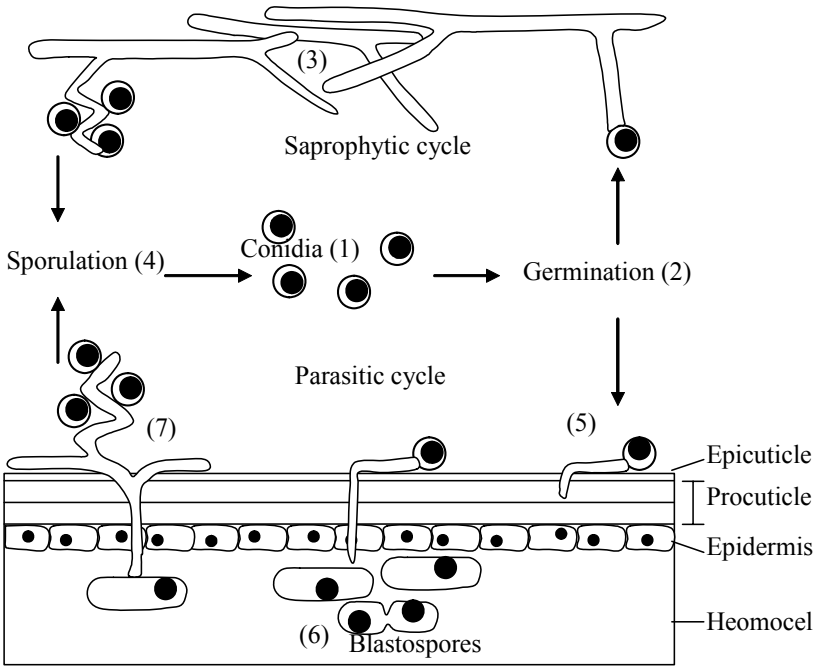

Figure 1-2. Life cycle of *Beauveria bassiana*: Metabolically dormant conidia (1); germination and production of hyphae in response to favorable growth conditions (2); mycelial growth on decaying plant matter (3); production of conidiogenic structure and formation and dispersal of new conidia (4); penetration of host cuticle by germ tube (5); fungus multiplies in heomocel as a single-celled blastospore (6). When nutrients are depleted, *B. bassiana* exits the cadaver as hyphae and begins the process of conidia production (7).

Table 1-2. Major chemical mediators of activated mast cells.

Mediator		Function
Vasoactive amine	Histamine	Irritates nerve endings, mucous secretion vasodilatation, bronchial and intestinal constriction
Proteases	Tryptase	Cleaves fibrinogen, activates collagenase, tissue damage
	Chymase	Converts angiotensin I to angiotensin II, degradation of epidermal basement membrane
Chemotaxins	ECF-A	Eosinophil chemotaxis factor
	NCF-A	Neutrophil chemotaxis factor
Lipid mediators	Platelet-activating factor	Aggregation and degranulation of platelets, pulmonary smooth muscle constriction
	Prostaglandin D2	Vasodilator, bronchial constriction, neutrophil chemotaxis
	Leukotriene C4	Vasodilator, prolonged bronchial constriction
Cytokines	IL-3	Stimulates mast cell growth and histamine secretion
	IL-4	B-cell differentiation, mast cell growth factor, Th2 differentiation,
	IL-5	Eosinophil activator, B-cell activator
	IL-6	B-cell proliferation into plasma cells
	IL-13	Inhibits production and release of macrophage cytokines
	GM-CSF	Granulocyte-macrophage colony stimulating factor, pro-inflammatory effects

Table contains the majority of mediators produced by mast cells, it does not list all chemicals produced by mast cells nor does it include all the effects of each chemical listed.

Table 1-3. Fungal allergens

Allergen	Function	MW (kDa)	Ref, or ID
Alternaria alternata			
Alt a 1	Unknown	28	U82633
Alt a 3	Heat shock prot. 70	-	U87807
Alt a 4	disulfideisomerase	57	X84217
Alt a 5	Ribosomal prot. P2	11	X78222
Alt a 6	Enolase	45	U82437
Alt a 7	YCP4 protein	22	X78225
Alt a 10	Aldehyde dehydrogenase	53	X78227
Alt a 12	Ribosomal prot. P1	11	X84216
Cladosporium herbarum			
Cla h 3	Aldehyde dehydrogenase	53	X78228
Cla h 5	Ribosomal prot. P2	11	X78223
Cla h 6	Enolase	46	X78226
Cla h 7	YCP4 protein	22	78224
Cla h 8	Mannitol dehydrogenase	28	AY191816
Cla h 9	Vacuolar serine protease	55	AY787775
Cla h 12	Ribosomal prot. P1	11	X85180
Aspergillus fumigatus			
Asp f 2	Unknown	18	U56938
Asp f 3	Peroxisomal protein	37	U20722
Asp f 5	Metalloprotease	40	Z30424
Asp f 8	Prbosomal prot. P2	11	AJ224333
Asp f 10	Aspartic protease	34	X85092
Asp f 12	Heat shock prot. 90	90	(1)
Asp f 16	Unknown	16	AJ002026
Asp f 18	Vacuolar serine protease	34	(2)
Asp f 22w	Enolase	46	AF284645
Penicillium chrysogenum			
Pen ch 13	Alkaline serine protease	34	AF321100
Pen ch 18	Vacuolar serine protease	32	AF263454
Penicillium citrinum			
Pen c 19	Heat shock prot. P70	70	U64207
Pen c 22w	enolase	46	AF254643

Data was obtained in large part from(Milligen, 2006); (1) (Kumar et al., 1993), (2) (Shen et al., 2001).

Table 1-4. Cross reactive fungal allergens

Allergen	Cross-reactive allergen or species
Enolase	Alt a 6, Asp f 22w, *C. albicans* , Cla h 6, *S. cerevisiae*
Aldehyde dehydrogenase	Alt a 10, Cla h 3
Heat shock protein	Alt a 3, Asp f 12, *C. albicans*, *C. herbarum*, Pen c 1
Peroxiaomal membrane protein	Asp f 3, *C. boidinii*, Mal f 2, Mal f 3, Pen c 3
Ribosomal protein P2	Alt a 5, Asp f 8, Cla h 4
Fibrinigen binding protein	Asp f 2, *C. albicans*
YCP4	Alt a 7, Cla h 5, *S. cerevisiae*
Vacuolar serine Protease	Asp f 18, Asp n 18, Pen ch 18, Pen o 18

Table data was obtained in large part from (Kurup and Banerjee, 2000)

CHAPTER 2
ALLERGENICITY[1]

Introduction

Microorganisms are currently under intensive study for use as biopesticides (Meikle et al., 2001; Shah and Pell, 2003). Several fungal species including *Metarhizium anisopliae*, *Verticillium lecanii*, and *B. bassiana* are being used as biocontrol agents for a number of agricultural and nuisance pests (Lecuona et al., 2001; Liu et al., 2003; Reithinger et al., 1997). Strains of *B. bassiana* have been licensed for commercial use against whiteflies, aphids, thrips, and numerous other insect and arthropod pests. *B. bassiana* fungal formulations are being spread onto a range of vegetables, melons, tree fruits and nuts, as well as organic crops. As alternatives to chemical pesticides, these agents are naturally occurring and are considered to be non-pathogenic to humans, although a few cases of *B. bassiana*-mediated tissue infections have been reported (Henke et al., 2002; Kisla et al., 2000).

Airborne mould spores are widespread, and many have been identified as inhalant allergens eliciting type I hypersensitive reactions in atopic individuals (Aukrust et al., 1985; Beaumont et al., 1985b; Chiu and Fink, 2002; Kurup et al., 2000b; Kurup et al., 2002). Common allergenic moulds include the anamorphs of Ascomycetes including many species within the *Alternaria*, *Aspergillus*, and *Cladosporium* genera (Banerjee et al., 1998; Banerjee and Kurup, 2003; Horner et al., 1995; Kurup et al., 2000a; Kurup et

[1] The text of Chapter 2 of this dissertation is a reprint (in part or in full) of the material as it appears in Clinical and Molecular Allergy (2005, Volume 3:1)

al., 2003). The genes encoding numerous fungal allergens have been isolated, and their protein products expressed and characterized. Purified fungal allergens have been shown to be bound by human IgEs and to elicit allergic reactions in atopic individuals using skin prick tests. Patients with mould allergies often display IgE-mediated responses to multiple fungi, a phenomenon typically thought to result from the presence of common cross-reactive antigen(s) (Aalberse et al., 2001; Aukrust and Borch, 1985; Gupta et al., 2002; Horner et al., 1995), although parallel independent sensitization to multiple fungal allergens can also occur. In this regards, identification of genus and/or species-specific antigens would provide useful tools in differentiating allergic reactions due to primary sensitization and those mediated by cross-reactive epitopes.

In the present study, we demonstrate that *B. bassiana* crude extracts contain numerous allergens recognized by human serum IgEs. The allergens were proteinaceous in nature, and immunoblot inhibition experiments revealed the presence of shared epitopes between *B. bassiana* and several other common fungal moulds. Potential *B. bassiana*-specific allergens were also identified, including a strongly reactive 35 kDa protein band. Intradermal skin testing using *B. bassiana* extracts resulted in allergenic reactions in several individuals, including some who have had occupational exposure to the fungus.

Material and Methods

Strains and Cultures

Beauveria bassiana (ATCC 30517) was grown on Sabouraud dextrose + 1% yeast extract or potato dextrose (PD) media on either agar plates or in liquid broth. Plates were incubated at 26^{o} C for 10–12 days and conidia were harvested by flooding the plate with sterile ddH_2O containing 0.01% Tween 20. Liquid cultures were inoculated with conidia

harvested from plates at 0.5–1x10^5 conidia/mL; 0.1 mL of conidia suspension per 100 mL culture media.

Extract Preparation

Lyophilized protein extracts of *Alternaria alternata* (lot# XPM1–X11), *Aspergillus fumigatus* (lot# XPM3–D13–15), *Candida albicans* (lot#XPM15–D16–23.35), *Cladosporium herbarum* (lot# XPM9–F6–1–23.85), *Epicoccum purpurascens* (lot#XPM29–D3–19.65), and *Penicillium notatum* (lot# XPM19–D4–16.8) were acquired from Greer Laboratories inc., (Lenoir, NC). Extracts were resuspended in TE (40 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final concentration of 2 mg/mL. *B. bassiana* was grown in Sabouraud's broth containing 1% yeast extract with aeration at 25ºC for 3–5 d. Fungal material (mixture of hyphae and blastospores) was harvested by centrifugation and freeze-dried. Cells were resuspended in TE containing 0.1% phenylmethylsulfonyl fluoride (PMSF) and homogenized using a bead-beater apparatus.

Precipitations

Crude extracts of *B. bassiana* were subjected to three successive precipitations before use in Western blots.

1) Acetone precipitation: *B. bassiana* extracts (50 mL) were mixed with 8x volume (400 mL) of acetone (kept at -20°C), with rapid stirring, and incubated overnight at -20˚C. The precipitate was collected by centrifugation (30 min, 4000 x g), and the pellet was air dried (10 min) before being resuspended in TE containing 0.1% PMSF.

2) Streptomycin precipitation (removal of DNA): Streptomycin sulfate (5 mL of 10% solution) was added drop wise to acetone precipitated *B. bassiana* extracts (40 mL) at 4˚C with rapid stirring. Samples were incubated for an additional 30 min on ice before

being centrifuged (15 min, 10,000 x g) in order to remove the precipitate. Proteins in the resultant supernatant were precipitated using ammonium sulfate.

3) Ammonium sulfate percipitation: The proteins present in the streptomycin sulfate treated supernatant were precipitated using ammonium sulfate (75%, final concentration). Saturated ammonium sulfate (120 mL) was added drop wise to the *B. bassiana* extract (40 mL) at 4˚C with rapid stirring. The solution was allowed to stir overnight at 4˚C and precipitated proteins were harvested by centrifugation (30 min, 100,000 x g). The protein pellet was resuspended in TE containing 0.1% PMSF (40 mL) and extensively dialyzed against the same buffer before use.

SDS-Polyacrylamide gel electrophoresis (PAGE): Protein samples (30–40 μg) were analyzed by SDS-PAGE (12% Bis-tris gel, Invitrogen, Carlsbad, CA) using standard protocols. Gels were stained with Gelcode blue stain reagent (Pierce, Rockford, IL) and subsequently de-stained with ddH_2O.

Western Blotting

Protein samples were separated under reducing conditions using 12% Bis-tris polyacrylamide gels (Invitrogen Mops system) and transferred to polyvinylidene-fluoride (PVDF) membranes (Invitrogen) as described. Immunoblot experiments were performed using individual and pooled human sera as the primary antibody solution as indicated. Typically, sera were diluted 1:5 with Tris-HCl buffered saline (TBS) containing 5% dry milk + 0.1% Tween–20. IgE-specific reactivity was visualized using a horseradish peroxidase (HRP) conjugated goat anti-human IgE (polyclonal) secondary antibody (BioSource International, Los Angeles, CA). Membranes were washed with TBS

containing 0.1% Tween–20 and bands were visualized using the Immuno-Star HRP detection system (Biorad, Hercules, CA).

Enzyme Treatments

The ammonium sulfate fraction of *B. bassiana* crude extracts was treated with Protease K (ICN-Biomed, Aurora, OH) following standard protocols. Typically, samples (36 μL) were incubated with 4 μL Proteinase K solution (10 mg/mL in 50 mM Tris-HCl, pH 7.5) for 2 hr at 37˚C before analysis. Samples were also treated with endoglycosidase-H (EndoH, New England Biolabs, Beverly, MA) and peptide: N-Glycosidase F (PNGaseF, New England Biolabs) according to the manufacturer's recommendations. For EndoH and PNGaseF treatments, samples (36 μL) were denatured in 4 μL 10x denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) at 100˚C for 10 min prior to the addition of the EndoH (5 μL of 10 x G5 Reaction Buffer, 50 mM sodium citrate, pH 5.5) and PNGaseF reaction buffers (50 mM sodium phosphate pH 7.5) and enzymes (5 μL), respectively. Reactions were incubated at 37˚C for 2 hr before being analyzed by SDS-PAGE and Western blotting.

Immunoblot Inhibition

IgE binding to *B. bassiana* proteins were competed with proteins of other fungal extracts. SDS-PAGE resolved *B. bassiana* proteins were electroblotted to PVDF membranes as described above. Membranes were blocked with TBS containing 5% dry milk + 0.1% Tween–20 and strips were incubated with pooled human sera (1:5 v/v in same buffer) containing 100–500 μg of the indicated fungal crude protein extract.

Skin Sensitivity Profiles to Fungal Extracts

Patients were tested with 9 common fungal extracts for allergy diagnosis using a skin prick assay. The following extracts were obtained from ALA-Abello (Round Rock, TX); *Alternaria tenius*, *Aspergillus fumigatus*, *Cephalosporium* (*Acremonium strictum*), Curvularia spp. *Bipolaris*, *Epicoccum nigram*, *Fusarium* spp., *Helminthosporium sativum*, *Hormodendrum horde*, *Penicillium chrysogenum* (formally *P. notatum*). Extracts were tested using a 1:10 dilution of 20,000 PNU/mL stock solution, and skin sensitivity was recorded on a relative scale from 0–4 reflecting the size of induration or weal (4 representing the highest reactivity). Histamine (0.1 mg/mL), which was used as a control, typically produced a reaction scored of 3.

Intradermal Skin Testing

B. bassiana crude extracts were prepared as described above but were extensively dialyzed against 0.15 N NaCl and filtered through a 0.22 μm filter before use. Subjects were given intradermal injections of 0.1 mL crude extract ranging in concentration from 0.01–1 mg/mL. Control injections included saline and histamine (0.1 mg/mL). Allergenic reactions were allowed to develop for 15–30 min before the height and width of the reactions were recorded.

Results

Identification of IgE Reactive Bands

An ammonium sulfate fraction of *B. bassiana* proteins was resolved on SDS-PAGE (Figure 2-1, lane B) and transferred to PVDF membranes as described in the Materials and Methods. Membranes were probed with sera from individual patients who were reactive to various moulds (Table 2-1), which was pooled and used to demonstrate IgE reactivity against antigens present in *B. bassiana* extracts (Figure 2-1). Serum mix-I

represents pooled sera derived from patients E, J, K, L, and M, as well as three additional patients that were only tested (skin prick) against Aspergillus and Penicillium, displaying test scores of 3–4 for each. These data demonstrate human IgE binding of antigens present in *B. bassiana* extracts. Initial blots showed 12–15 distinct reactive protein bands, ranging in molecular mass from 12 kDa to >95 kDa (under denaturing conditions); with the most prominent bands located around 64, 45, and 35 kDa. Control experiments omitting either the primary or secondary antibody incubation steps resulted in complete loss of signal. Proteinase K digestion of samples also resulted in loss of all signal (Figure 2-1, lane 4), indicating the proteinaceous nature of the IgE reactive bands. Since the carbohydrate moieties of several protein allergens are known to play a role in their allergenicity and even cross-reactivity (Aalberse et al., 2001; Ebo et al., 2004; Hemmer et al., 2004), samples were treated with the deglycosylating enzymes EndoH and PNGaseF. Control experiments incubating samples in the EndoH denaturing buffer without any enzyme altered the IgE-reactive signals (Figure 2-1, lane 5), however, samples treated with EndoH did not appear any different than control reactions (Figure 2-1, lane 6). Similar results were obtained in PNGaseF digests (data not shown). These data appear to indicate that the *B. bassiana* IgE-antigen profiles observed on Western blots are proteins with minimal contributions due to glycosylation.

Immunoprint Analysis of *B. bassiana*: Reactivity with Individual Sera

In order to determine the variation and distribution of serum IgEs reactive to *B. bassiana* extracts, individual sera from patients displaying mould allergies (Figure 2-2, lanes A–G) as well as random sera from the general population (Figure 2-2, lanes H–M) were used as probes for western blots (Figure 2-2). The reactivity of pooled sera from patients A–G (termed serum Mix-II) is also shown (Figure 2-2, lane 2). Skin prick test

results for patients A–G are shown for comparative purposes (Table 2-1) and represent the clinically determined reactivity of each patient to extracts of the tested fungal species. Patients (A–G) were selected based on skin prick reactivity to at least 4 different fungi with scores of 2 or greater. Identical concentrations of *B. bassiana* extract (40 μg) were resolved by SDS-PAGE, blotted to PVDF membranes, and the lanes were cut into separate strips. Each strip was treated with a 1:5 dilution of each respective serum as described in the Materials and Methods (Figure 2-3, lane 2 is the sera pool). A total of 16 individual sera were tested, with the sera from three patients displaying no IgEs reactive to proteins present in the *B. bassiana* extracts. The results for the remaining 13 sera are shown in Figure 2-2. The data show a large individual variation in serum IgEs capable of binding epitopes present in *B. bassiana* extracts, both in terms of banding distribution and the intensity of the reaction. No correlation was observed between measurements of total IgE and the observed binding to *B. bassiana* antigens. Some patients displayed strong reactions to multiple bands, whereas others to a more limited set of epitopes. Distinct strongly reactive bands ranging from 40 kDa to approximately 200 kDa could be seen in sera A, E, and to a lesser extent L. A strongly reactive 35 kDa band was visible in sera C, G, E, and L. Several sera displayed IgEs that bound to only a limited set of 2–3 antigens (C, F, G, weak bands in B, I, J, K, and M). Blots probed with one serum (H) resulted in a large smear ranging from ~30 kDa to 55 kDa. The reason for the observed smear is unknown and efforts to distinguish separate bands by manipulating the concentrations of either sera or extract were unsuccessful. A number of bands (based upon molecular mass) appeared to be common to several sera including proteins of approximately 35, 42–

48, and 60 kDa. A number of antigens of high molecular weight (~100–200 kDa) were also visible; however, the resolution in this range on the Western blots is poor.

Intradermal Skin Testing

A total of ten individuals were tested for allergenic reactivity to *B. bassiana* crude extracts using an intradermal delivery procedure. Data using 1 mg/mL *B. bassiana* crude extract and histamine controls are presented in Table 2-2. Seven out of the ten individuals (ID #s, J–O, and Q) displayed skin reactivity reactions to the *B. bassiana* extracts (Table 2-2, also see corresponding Western blot results for individuals J, K, L, and M; Figure. 2-2). It is interesting to note that 4 (J–M) of 5 individuals (J-M and S) that have had occupational exposure to *B. bassiana* displayed skin reactivity as well as bands on western blots. A preliminary correlation was observed between the *B. bassiana*/histamine reaction and the *in vitro* reactivity of individual sera in Western blots. Individuals J, K, and M, displayed *B. bassiana*/histamine control ratios <1, also showed weak bands in western blots (Figure 2-2), whereas individual L who had a *B. bassiana*/histamine ratio = 1.65, reacted against numerous epitopes in the extract and with a higher intensity.

Cross-Reactivity among Different Fungi

In order to determine the extent of cross-reactivity of *B. bassiana* antigens with other fungi, immunoblot inhibition experiments were performed. Identical concentrations of *B. bassiana* crude extract (40 μg) were resolved by SDS-PAGE, blotted to PVDF membranes, and lanes were cut into separate strips. Each strip was treated with a 1:5 dilution pooled sera (serum mix-II) as the primary antibody supplemented with concentrations of fungal crude extracts as described in the Materials and Methods. Figure 2-3 shows Western blots in which the binding of human IgEs to antigens present

in *B. bassiana* extracts were competed with: excess crude extracts from *Alternaria alternata* (Figure 2-3, Lanes 3,4), *Aspergillus fumigatus* (Lanes 5,6), *Cladosporium herbarum* (Lanes 7), *Epicoccum purpurascens* (Lane 8), *Penicillium notatum* (Lane 9), and *Candida albicans* (Lane 10). There was complete loss of all signals using 2-fold excess *B. bassiana* extract as the competitor (data not shown). These data indicate that while *B. bassiana* possess many epitopes in common with several other fungi, notably *Alternaria* and *Penicillium*, a 35 kDa major reactive band was not inhibited by any extract tested.

Discussion

Although it is well known that fungi are important triggers of respiratory allergies, the potential allergenicity of entomopathogenic fungi used in biocontrol has largely been untested. Aerobiological surveys conducted in the Netherlands in the late 1980s comparing the environmental concentrations of fungal spores with their allergenicity, reported that although *B. bassiana* represented less than 0.1% of the airborne fungal "flora", it elicited the most severe allergenic skin test response of all fungal species tested (Beaumont et al., 1985a; Beaumont et al., 1985b; Beaumont et al., 1985c). In rural areas, the use of fungi in agricultural pest management practices can greatly increase the potential for human exposure to these agents. Likewise, in urban settings, the commercialization of fungal products for household use may result in a much wider problem since indoor air concentrations of the moulds can greatly increase. For these reasons, an examination of the allergenic potential of *B. bassiana* is imperative.

The present study demonstrated the allergenic potential of *B. bassiana* directly by intradermal skin testing of individuals and *in vitro* by revealing the presence of serum IgEs capable of binding allergens present in fungal crude extracts. Over 20 different IgE

binding proteins were observed using Western blots probed with sera from patients displaying mould allergies. Results using individual sera revealed a wide variation in IgE-binding proteins between sera, although several common bands, including a protein with an apparent molecular mass of 35 kDa, were visible among the sera of several patients.

Our *in vitro* observations were confirmed by intradermal skin testing on individuals using *B. bassiana* extracts. While the testing sample population was small, the results indicated that our extracts were able to elicit allergic reactions in individuals, including some that have had occupational exposure to the fungus. Concentrations of ~1 mg/mL of *B. bassiana* extracts were required to elicit indurations equivalent to 0.1 mg/mL histamine in most individuals, indicating the possibility of potent allergens in the *B. bassiana* extract. Interestingly, not all individuals specifically exposed to *B. bassiana* displayed allergic reactions and individuals J, K, and M (who did display mild allergic reactions, Table 2-2) did not react to the 35 kDa protein based upon Western blotting results (Figure 2-2). We do not, however, have any quantifiable index of exposure for the individuals in our sample and any interpretations should be made with some caution.

Numerous studies have revealed the presence of cross-reactive proteins among fungal species between genera (Aalberse et al., 2001; Aukrust and Borch, 1985; Gupta et al., 2002; Horner et al., 1995; Simon-Nobbe et al., 2000; Vieths et al., 2002; Weichel et al., 2003). In our experiments, (excess) crude extract from a test organism was added during the primary antibody (human sera) incubation. Common or shared epitopes between *B. bassiana* and the test fungus would result in a loss of signal due to competition for reactive IgEs. However, IgEs reactive to *B. bassiana*-specific antigens

would not be affected, resulting in no change in the corresponding reactive bands on a Western blot. Loss of a signal would indicate that a homolog or shared epitope (IgE-reactive) exists between the two fungal species, implying that primary sensitization by one organism can result in an allergic reaction when exposed to the homologous allergen of another organism. Competitive immunoblot inhibition experiments revealed significant epitope homology between *B. bassiana* and several clinically important fungi responsible for IgE-mediated allergic reactions in atopic individuals. Thus, an allergic reaction to B. bassiana exposure may arise in patients sensitized to other fungi. Extracts from *A. alternata* and *E. purpurascens* almost completely competed with antigens present in the *B. bassiana* extract with the notable exception of the ~35 kDa allergen. Competition experiments using *A. fumigatus*, *C. herbarum*, *C. albicans*, and *P. notatum* extracts also indicated the presence of many shared epitopes, although distinct (non-competed) IgE-binding *B. bassiana* proteins of 35 kDa, 64 kDa, and >200 kDa molecular mass were detectable. These proteins, particularly the 35 kDa antigens may represent *B. bassiana*-specific allergens. Experiments are underway to characterize the 35 kDa allergen, which may lead to a diagnostic assay for *B. bassiana* sensitization. Finally, our analysis of potential *B. bassiana* allergens was limited to cell extracts grown under specific conditions and did not include the culture filtrate. Extracellular proteases, an important class of fungal proteins that can elicit allergenic reactions, have been characterized form a number of fungal species (Chou et al., 2003; Gupta et al., 2004; Nigam et al., 2003; Shen et al., 2001), and are likely to be present in *B. bassiana*. A careful examination of culture growth conditions is also warranted in order to provide a standardized reagent for testing purposes.

Conclusion

Although *B. bassiana* holds promise as an arthropod biological control agent, there have been few reports on the allergenic potential of these organisms. Identification of *B. bassiana*-specific allergens can lead to diagnostic methods for determining sensitization to this organism and may provide a rational basis for allergen attenuation in order to yield safer biocontrol products. The observed cross-reactivity among proteins of *B. bassiana* and the fungi tested, highlight the importance of considering the possibility that multiple fungal sensitivity can occur due to exposure to a single fungus. Further testing should be performed to determine the scope, severity, and range of allergenic reactions to *B. bassiana*.

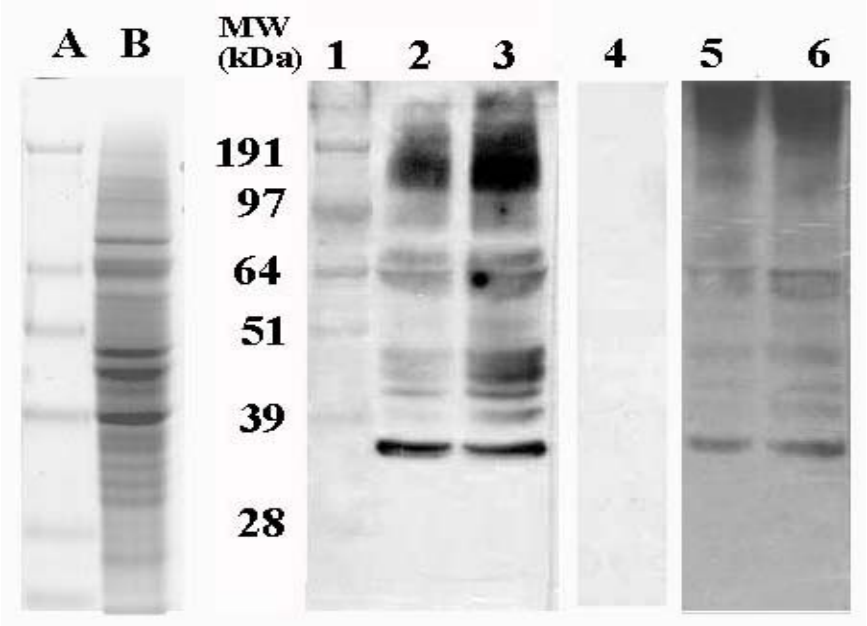

Figure 2-1. SDS-PAGE and Immunoblot analysis of *B. bassiana* crude extracts. SDS-PAGE, Gelcode blue stained, lanes A) 5 µg protein standards, and B) 40 µg *B. bassiana* crude extract. Immunoblots probed with pooled sera mix-I (patients displaying mould allergies), lanes 1), 5 µg protein standards, 2) 20 µg *B. bassiana* crude extract, 3) 40 µg crude extract, 4) 40 µg crude extract, proteinase K treated, 5) 40 µg crude extract, denaturing buffer control (no EndoH), 6) 40 µg crude extract, EndoH treated.

Table 2-1. Allergic profile of patients A–G, obtained by skin testing

Patient no.	Individual Reactivity* to Fungal Extracts†								
	Alt	Asp	Cep	Cur	Epic	Fusa	Helmin	Hormo	Pen
A	3	2		3	2	2			3
B	3	2		2	3	2	2	2	
C	4			3		2			
D			2	2		2		2	2
E	3	2		3	2	3	3		
F	4	1	1	2	4		2		
G	3			4	3		2		

*Skin test score is registered 0–4 with 4 representing the most reactivity. † Abbreviations are: Alt-Alternaria, Asp-Aspergillus, Cep-Cephalosporium, Cur-Curvularia, Epic-Epicoccum, Fusa-Fusarium, Helmin-Helminthosporium, Hormo-Hormodendrum, Pen-Penicillium.

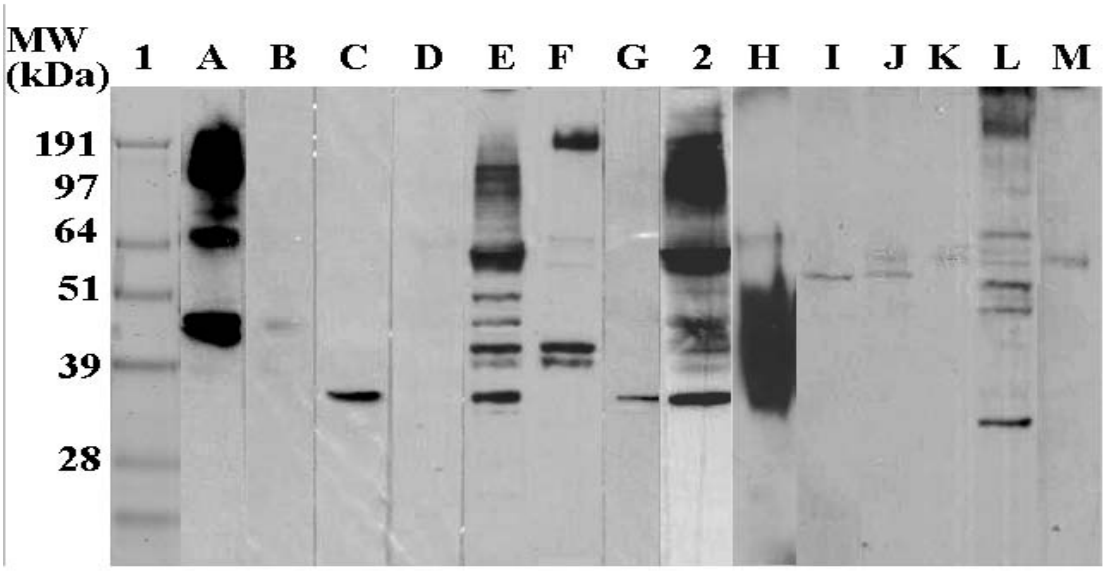

Figure 2-2. Immunoblot analysis of *B. bassiana* extracts (40 µg crude extract/strip) probed with individual sera. Lane 1) 5 µg protein standards, 2) pooled sera mix-II (patients displaying mould allergies). Lanes A)–G) membranes strips treated with individual sera from sera mix-II. Lanes H)–M) membrane strips probed with individual sera randomly obtained from the general public.

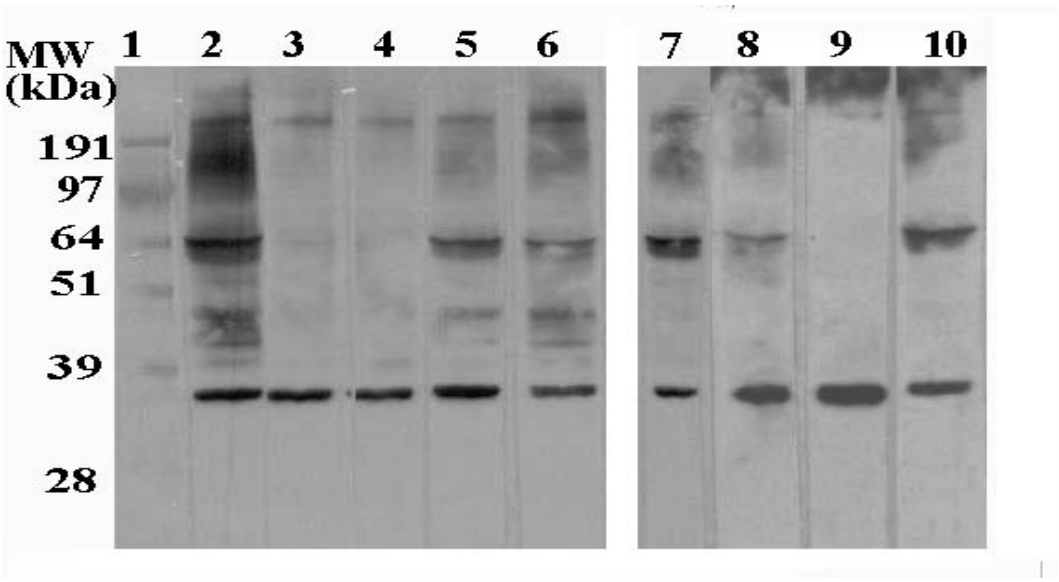

Figure 2-3. IgE immunoblot inhibition with fungal extracts. *B. bassiana* protein strips (40 µg crude extract) were blocked and incubated with mix containing (500 µl) pooled sera (mix-II) 1) Seablue standard, 2) no additions, 3) 40 µg *A. alternata* crude extract, 4) 400 µg *A. alternata*, 5) 40 µg *A. fumigatus*, 6) 400 µg *A. fumigatus*, 7) 400 µg *C. herbarum*, 8) 400 µg *C. albicans*, 9) 400 µg *E. purpurascens*, and 10) 400 µg *P. notatum* protein.

Table 2-2. Intradermal skin test

Patient ID	Histamine control (0.1mg/mL)		*B. bassiana* Extract (1mg/mL)		*B. bassiana*/Histamine (mm²/mm²)
	Induration	Erythema	Induration	Erythema	Induration ratio
J[3]	7x6	12x16	8x8	12x13	0.65
K[3]	20x15	55x50	13x12	14x13	0.52
L[3]	11x10	16x33	13x14	26x28	1.65
M[3]	15x16	36x44	10x12	10x12	0.30
N	16x14	38x58	10x11	21x17	0.49
O	21x16	39x59	9x8	18x21	0.21
P	15x17	44x45	5x4	5x4	0.08
Q	15x14	36x38	9x12	10x13	0.51
R	15x15	55x38	4x4	11x13	0.07
S[3]	20x19	38x43	4x4	4x4	0.04

[1]In all instances saline control produced an Induration of 3–4 x 3–4 mm.
[2]Induration and erythema values are recorded in mm.
[3]Individual with occupational exposure to *B. bassiana*.

CHAPTER 3
MOLECULAR AND IMMUNOLOGICAL CHARACTERIZATION OF PUTATIVE *B. bassiana* ALLERGENS

Introduction

Allergic diseases represent a growing human health problem, affecting up to 25% of individuals living in industrialized nations (Chiu and Fink, 2002). Both in- and outdoor populations of filamentous fungi are a major cause of human allergies, and can in some cases, lead to severe allergic disease (Kurup et al., 2000b). Common clinical symptoms of atopic allergy include sneezing, rhinitis, shortness of breath, and asthma. Asthma is a chronic respiratory disease that afflicts over 17 million Americans, and is responsible for 5,000 deaths annually (O'Hollaren, 2006). Some 30% of asthma cases can be attributed to exposure and sensitization to filamentous fungal allergens (Kurup et al., 2002; Vijay and Kurup, 2004; Wuthrich, 1989).

Beauveria bassiana is an entomopathogenic fungi currently used as a biological control agent against agricultural insect pests (Shah and Pell, 2003). *B. bassiana* is considered non pathogenic to vertebrates and has not been deemed a potential health or environmental hazard (EPA, 2000). Research presented in this thesis as well as by others has shown, however, that *B. bassiana* is capable of initiating an allergic response in humans; and applications of this fungus should take into account potential health concerns regarding eliciting allergenic reactions.

A volumetric assay of allergens in the 1980's revealed that although the environmental concentration of *B. bassiana* spores was very low, the allergic response

was severe (Beaumont et al., 1985a; Beaumont et al., 1985c). Performing skin prick assays on patients with mold allergies, *B. bassiana* was shown to elicit on of the strongest reactions relative to the other fungal species in the study. In research presented as part of this thesis we have demonstrated that human IgEs, derived from patients displaying allergies to molds, react with several proteins produced by *B. bassiana*. Furthermore, many of these proteins were cross reactive with allergens of other major allergenic fungi (Westwood et al., 2005).

The majority of fungal allergens are proteins of unknown function; however, the biochemical activities of a number of allergens have been characterized. These typically fall into several classes including metabolic enzymes, proteases, and enzyme inhibitors (Stewart et al., 1993). An enzyme or protein identified as an allergen in one species of fungus is often found to be allergenic when identified in other species due to similarities in structure and/or function. In many cases, structural similarities between the proteins of two species are close enough to be recognized by the same specific IgE antibodies, leading to cross-reactivity. Aldehyde dehydrogenase has been identified as a major allergen in both *Alternaria alternata* (Alt a10) and *Cladosporium herbarum* (Cla h3) (Achatz et al., 1995). Enolase (2-phosho-D-glycerate hydrolase), a glycolytic enzyme responsible for the production of phosphoenolpyruvate, has been identified as an allergen of not only *C. herbarum* and *A. alternata* but of several other fungal species as well (Simon-Nobbe et al., 2000).

Here we report the identification of four *B. bassiana* proteins as potential allergens. Full length cDNA and genomic nucleotide sequences of the four genes were determined. Similarity search results of the translated open reading frames of the proteins coded by

the genes have led to their putative designation as follows; BbEno1, an enolase; BbF2, major allergen of *Aspergillus fumigatus*; BbAld, aldehyde dehydrogenase; and BbHex, acetyhexoseaminadase. The cDNA sequences of all four proteins were used to design primers for subcloning of the genes into *E. coli* expression vectors. All four proteins were successfully expressed as recombinant proteins in *E. coli*. Two of these proteins, a suspected enolase (BbEno1), and a suspected aldehyde dehydrogenase (BbAld) reacted with human IgEs derived from patients displaying mold allergies.

Materials and Methods

Strains and Media

B. bassiana (ATCC 90517) was maintained on potato dextrose agar (Difco, MI) at 26˚C. For RNA extraction, driver and tester culture where grown on selective metabolic media; media and extraction perform as outlined in (Holder, 2005). *E. coli* strains used for cloning and heterologous protein expression included: TOPO Top 10, chemical competent cells (Invitrogen, CA); BL21 Rosetta (DE3), harboring the pRARE plasmid (Novagen, Darmstadt, Germany). *E. coli* cloning and expression strains were grown and/or maintained in Luria-Bertani (LB) or on LB Agar (LBA) (Difco, Detroit, MI), at 37˚C.

RACE PCR

Full length gene sequences of the *B. bassiana* genes were obtained by RACE PCR technology (rapid amplification of cDNA ends). The SMART RACE cDNA Amplification kit (Clontech, CA) was used according to manufacturer instructions. Template mRNA was extracted from *B. bassiana* grown on minimal medium with 1% (w/v) glucose (0.4 g/L KH_2PO_4, 1.4 g/L Na2HPO_4, 0.6 g/L $MgSO_4.7H_2O$, 1.0 g/L KCl, 0.25 g/L NH_4NO_3, 0.01 mg/L $FeSO_4$ and 10 g/L sterilized cuticle) and 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) (Holder, 2005). 5' and 3' primers used for RACE are listed on Table 3-1

Cloning

Primers where designed to clone the entire cDNA gene and to incorporate restriction sites for extraction and insertion into an expression vector (Table 3-1). An NdeI restriction (catatg) site was incorporated into the forward primer and an EcoRI site (gaattc) was incorporated into the reverse primer. PCR products were cloned directly into TOPO 2.1 using TOPO TA cloning system and transformed into TOPO Top 10 *E. coli* cells (Invitrogen, Carlsbad, CA).

The *B. bassiana* genes where subcloned from the TOPO 2.1 constructs into the *E. coli* pET43.1a (Novagen, Darmstadt, Germany) expression system using the NdeI and EcoRI restriction sites in the clones and vector. For expression, pET43.1a containing *B. bassiana* genes and *amp* resistance gene were transferred to an *E. coli* BL21 and expression strain, Rosetta (DE3) (Novagen, Darmstadt, Germany). The host strain harboring the pRARE plasmid which contains the genes for production of the rare codons; proL, leuW, metT, argW, thrT, glyT, tyrU, thrU, argU, and ileX. Thus, final expression cells contain two plasmids, pRARE and the pET construct.

Expression

The four *B. bassiana* proteins were expressed using an *E. coli* T7 polymerase based recombinant system. Overnight cultures of *E. coli* BL21 harboring pRARE along with each respective pET43.1a-based construct were grown in 3 mL of LB (Amp 50 μg/mL, Cam 12 μg/mL) at 37˚C with aeration. 5 mL of fresh media was inoculated with 100 μL

of the overnight culture, and samples were incubated at 37˚C with aeration to OD_{600} of 0.6–0.8 (2–3 hrs). Initial growth aliquots were taken and stored as uninduced samples. IPTG (Isopropyl-ß-D-thiogalactopyranosid) was then added to the remaining culture to a final concentration of 1.5 mM; cultures were returned to the incubator for an additional 2 hours. For extract preparation, cells were harvested by centrifugation (10,000 x g, 10 min) and the resultant pellet resuspended in 0.5 volumes TE (40 mM Tris, 1 mM EDTA, 0.01% phenylmethylsulfonyl fluoride (PMSF)). Cells were lysed by sonication (3 x 30 sec) on ice. Sonicated samples were centrifuged (10,000 x g, 10 min) and separated into soluble and pellet (containing potential inclusion bodies) fractions. Both fractions were denatured with 1x LDS loading dye and boiled for 5–10 min prior to separation by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). Samples (15 μL) were analyzed by SDS-PAGE using the Invitrogen NUPage, Mops system (12% Bis-tris gel) using the manufacture's recommended protocols. Gels were stained with Coomasie Blue R250 followed by destaining with 10% methanol, 10% acetic acid solution.

Western Blot and Immunodetection

Protein samples were electrophoresed under reducing conditions using Bis-Tris SDS-PAGE 10–12% gels (Invitrogen NuPAGE, Mops system), followed by electroblotting to polyvinylidene-fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Membranes were incubated in blocking buffer (TBST (25 mM TBS, 0.1% Tween–20), 10% dry fat free milk), either individual or pooled human sera as the primary antibody solution. Typically, sera were diluted in blocking buffer and incubated with membranes overnight at 4–8˚C with gentle agitation. Membranes were washed 3x using 50 mL TBST for 15 min. Binding of human IgEs was visualized using a horseradish

peroxidase (HRP) conjugated goat anti-human IgE (polyclonal) secondary antibody (BioSource International, CA). Membranes were incubated in secondary antibody (diluted 1:10,000 in blocking buffer) for 1 hr at room temperature, with gentle agitation. After secondary antibody incubation membranes were washed 3x using 50 mL TBST. Bands were visualized using the Immuno-Star HRP detection system (Bio-Rad, Hercules, CA).

Membrane staining was performed by 5 minute incubation in Ponceaus S (Sigma, St. Louis, MO) and destained for 2 minutes with ddH_2O.

Analysis Programs

Nucleotide manipulations were done using Vector nti, which was also used to generate figures showing nucleotide and amino acid sequences. Phylogenetic analyses of amino acid sequences were performed using ClustalW and SplitsTree. Initial sequence alignments where performed with ClustalW (Thompson et al., 1994). Alignment files (in Nexus format) were transferred to SplitsTree for analysis and construction of phylograms, with typical bootstrap parameters set to 1000 (Huson and Bryant, 2006).

Results

Cloning and Sequencing

EST (expressed sequence tag) panning and screening of a suppressive subtractive library (SSH) identified gene fragments of four potential allergens by sequence homology (Table 3-2) (Holder, 2005). The *B. bassiana* genes were designated as follows: *bbeno1*, similar to *Cladosporium herbarum* enolase Cla h 6; *bbf2*, similar to *Aspergillus fumigatus* major allergen Asp f 2; *bbald*, similar to *Cladosporium herbarum* allergen Cla h 3, an aldehyde dehydrogenase; and *bbhex*, with similarities to numerous fungal acetyl-hexosaminidase, including Pen ch 20.

Since the nucleotide fragments (200–900) represented only a portion of the entire gene sequence coding for each protein, full length sequences were obtained by two rounds of RACE PCR. Based upon the final assembled gene sequences, primers were designed incorporating the restriction site NdeI at the 5' end and EcoRI at the 3' end. Primers were designed for amplification of both genomic and cDNA sequences of each gene (Figure 3-1) (Table 3-2).

The genomic sequence of *bbeno1* consisted of 1548 bp from the start site to the stop codon and contained 4 introns (Figure 3-3). All four introns were between the lengths of 52–69 bp and are located in the first half of the gene. *bbeno1*, 1317 bp cDNA sequence, encodes a 47 kDa protein 438 amino acids in length. Blastx similarity searches of BbEno1 amino acid sequence against the NCBI protein database resulted in high similarity to the enolases of several different fungal species, including *Aspergillus fumigatus*, *Penicillium citrinum*, *Alternaria alternata*, and *Cladosporium herbarum*. These enolases are also known to be highly allergenic.

The genomic sequence of *bbf2* consisted of 845 bp from start site to the stop codon and contained one intron that began at bp 412 and was 59 bp in length (Figure 3-4). *bbf2* encodes a 28 kDa protein, 261 amino acids in length. Blastx similarity searches for BbF2 against the NCBI protein data bases identified sequence similarity to *Aspergillus fumigatus* major allergen Asp f 2. The function of the protein Asp f 2 is unknown.

The genomic sequence of *bbald* consisted of 1659 bp from start site to stop codon and contained two introns (Figure 3-5); the first 106 bp in length, started at bp 62, and the second, 59 bp in length, started at bp 568. *bbald* 1494 bp cDNA sequence encodes a 53 kDa protein, 497 amino acids in length. Blast similarity searches of BbAld against the

NCBI protein database revealed amino acid sequence similarities to the aldehyde dehydrogenases produced by several fungal species including *Alternaria alternata* and *Cladosporium herbarum*, both of which are known allergens.

The genomic sequence of *bbhex* consists of 1959 bp from start site to stop codon and contained no introns (Figure 3-6); translation results in a 72 kDa protein with an amino acid length of 652. Blastx similarity searches showed sequence similarity to several fungal N-acetylhexosaminidases one of which (Pen ch 20) is a major allergen of *Penicillium chrysogenum.*

Protein Expression

All four *B. bassiana* genes were subcloned into the pET43.1a expression vector as described in the Materials and Methods. The integrity of all the clones was verified by sequencing of the inserts. Initial attempts using the *E. coli* BL21 (DE3) (Novagen) yielded no visible expression of the proteins after inductions as determined by SDS-PAGE. The clones were then transformed into a BL21 *E. coli* strain containing pRARE, a plasmid that contains the genes for the expression of ten rare tRNAs (Novagen).

Expression experiments were conducted with a 2 hr induction period and samples analyzed by SDS-PAGE. Highly expressed protein bands were visible in lanes containing samples that were IPTG induced, with no highly expressed protein visible in the uninduced lanes (Figure 3-7). Note that the pET43.1a vector contains a collection of fusion tags (Nus-Tag, His-tag, and S-Tag), which were removed in the cloning of *B. bassiana* putative allergen genes, however in the "no insert" control, the combined length of the fusion tags is ≈1800 bp and results in the expression of a polypeptide of approximately 70 kDa (Figure 3-7, lane 10).

Induction of the BbEno1 clone by IPTG resulted in the production of two bands, the first having the expected mass of 47 kDa, and a second smaller band with a mass ≈45 kDa (Figure 3-7, lane 2). The BbF2 clone also appeared to produce two protein bands of ≈28 kDa (Figure 3-7, lane 4). Experiments were performed varying the induction time from 1 to 3 hrs and in all cases the expression of two proteins was apparent in the BbEno1 and BbF2 (data not shown).

Effect of Denaturing Conditions on Expressed Proteins

In order to determine whether the two bands observed during expression of BbEno1 and BbF2 was the result of cleavage of the intact protein during denaturation, protein aliquots were placed in PAGE sample buffer and boiled for various times. Fresh cultures were grown and induced (2 hrs with 1.5 mM IPTG), and aliquots were placed in 1x LDS loading buffer. Samples of all the four induced cultures were incubated at 95˚C for 1, 5, and 20 minutes to lyse cells and denature proteins prior to being analyzed on 12% PAGE gels (Figure 3-8), a similar experiment was conducted with a 5 minute incubation of time and increasing temperatures 95, 100, 110˚C (data not shown). The data revealed that denaturing conditions (boiling in sample buffer) results in the partial breakdown of some of the expressed clones visualized by the increasing intensity of a lower molecule weight band. Only a single band was visible in the induced lanes of BbEno1 and BbF2 when denatured (heated) for one minute at 95˚C, whereas increased time course of heating led to the appearance of a second BbAld band. The 72 kDa band produced by BbHex clone was not affected under the conditions tested (Figure 3-8).

IgE Reactivity

To test for allergenicity, the four recombinant *B. bassiana* proteins were separated by PAGE electrophoresis and electroblotted onto PVDF membranes. Membranes were

blocked with 10% milk in TBST and treated with human sera to test for IgE reactivity. Sera used were collected from 20 patients with known fungal allergies. Each serum was randomly assigned an alphabetical designation, and in most instances pooled was described alphabetically according to the sera it contained. Figure 3-9 shows two blots containing all four expressed proteins as well as a crude *B. bassiana* extract (positive control), that were probed with one of two sera pools containing serum from ten patients each, pools A–J and K–T. Each blot was treated with 200 μL of each serum diluted in 5 mL blocking buffer, with a final volume of 7 mL and the concentration of each serum being 1:35. HRP conjugated goat anti-human IgE was used to identify bands that had been bound by human IgEs. The blot probed with pool A–J revealed strong IgE binding of the two protein bands corresponding to BbEno1, as well as several reactive (background) *E. coli* bands (Figure 3-9). The *B. bassiana* crude extract reacted with a variety of IgEs present in the sera (Figure 3-9, lane 5 and 10). Since, a number of experiments resulted in the faint potential interaction of sera IgEs with BbF2 and BbAld experiments performed using smaller sera pools in which the concentration of any individual sera was increased.

Five sera pools were created each containing 1:5 dilutions of two sera each, designated as AB, CD, EF, GH and IJ. These pools were then used to probe blots containing BbEno1, BbF2, and BbAld, (BbHex was omitted due to the lack of even faint reactivity). Background bands were highly variable between pools and were the results of specific IgE interactions. Pool EF reacted strongly against *E. coli* proteins of similar molecular weight as BbEno1 and BbAld (Figure 3-10), and was therefore not used any

further. IgEs in pools AB and GH reacted against BbEno1. Human IgE binding to BbAld was also noted with pools AB and GH.

Since many recombinant proteins often form inclusion bodies when expressed in *E. coli*, soluble and pellet fractions were isolated for all four *E. coli* expressed *B. bassiana* proteins after IPTG induction. SDS-PAGE analyses of these fractions revealed, that all four *B. bassiana* proteins were located in the insoluble pellet fraction, and hence are likely localized within inclusion bodies (Figure 3-11).

Figure 3-12 presents three blots demonstrating the human IgE binding and specificity of BbEno1. Panel (ABCD) shows BbEno1 (unfractionated samples) sample treated with a pool of ABCD (1:10 dilution). The two bands corresponding to BbEno1 were bound by sera IgEs (Molecular mass range between 45–49 kDa). Panel (AB) of Figure 3-12 shows that there are no reactive bands present when serum (pool AB) is used to screen the uninduced sample or the soluble fraction. Panel (CD) of Figure 3-12 show a blot treated with human sera (pool CD) from individuals not sensitized (allergic) to BbEno1.

To confirm BbAld reactivity, 15 µL samples of BbAld pellet fraction were run on a 10%, 15 lane polyacrylamide gel (NuPAGE, Invitrogen); the thinner lanes and lower percent gel appeared to slightly improve the resolution of the protein bands. After electroblotting onto PVDF membranes, cut lanes were treated with sera pools as follows: AB, CD, GH, IJ, KJ, MN, OP, QR, and ST (1:5 dilutions), 9 pools total (Figure 3-13). These results demonstrated BbAld specific IgEs were present in four of the nine sera pool tested including pool AB.

A further experiment testing for the presence of sera IgEs capable of binding BbEno1 and BbAld was performed. Figure 3-14 shows the results of blots containing the pellet fraction of the four *B. bassiana* proteins, probed with individual serum A and B. Blots were compared to a membrane stained with Ponceaus S, which confirms the presence and efficient transfer of the proteins (Figure 3-14).

Phylogenetic Comparison

BbEno1 displays high sequence similarity to fungal enolases many of which are major allergens. All available fungal enolase sequences were gathered including both allergenic enolases as well as enolases not known to be allergenic in order to construct a phylogenetic comparison. The enolase from *Hevea brasiliensis* (rubber tree) was included in this comparison since it too is an allergen. Two additional non-fungal enolases were included as outlying sequences for rooting the tree. The non-fungal enolases included the enzymes from *Drosophila melanogaster* (fruit fly) and *Escherichia coli* (prokaryotic bacterium).

Enolase amino acid sequences where prepared for phylogenetic analysis by first running a ClustalX alignment (Thompson et al., 1994), and the resultant product saved in Nexus format to enable analysis by SplitsTree phylogenetic program (Huson, 1998). Data was analyzed and organized into rooted phylograms (Figure 3-15). Probabilities were calculated using a bootstrap value set to 1000.

Of the 21 fungal enolases, eight have been identified as allergenic including BbEno1. Known allergens are depicted by an asterisk in Figures 3-15. The positions of the allergens on the phylogram do not appear to be grouped or form any pattern, and they are equally distributed throughout the cladogram. It should be noted that enolases not

marked as allergens do not necessarily represent non-allergens, but reflect cases where allergenicity has not been reported.

Discussion

Several studies have demonstrated the potential of *B. bassiana* to elicit allergic reactions in humans (Beaumont et al., 1985a; Beaumont et al., 1985c; Westwood et al., 2005). In this study we have taken the next step toward a better understanding of the allergenicity of *B. bassiana*.

Allergic testing towards *B. bassiana* is not routinely performed and indeed there are no approved extracts for testing patients for *B. bassiana* allergy. Therefore, the serum collected was from patients that reacted to other fungi such as *Aspergillus*, *Alternaria*, and *Epicoccum*. Since many patients that display allergies are often sensitive to multiple fungi (Horst et al., 1990), it was hypothesized that within a population of patients with known fungal allergies there would exist individuals sensitive to *B. bassiana*, and that the cross-reactive nature of fungal allergenic epitopes would increase the likely hood of finding individuals allergic to *B. bassiana* proteins.

We have demonstrated that crude extracts of *B. bassiana* contained several proteins that reacted with human sera IgE. EST and SSH revealed four proteins that were highly similar to other fungal allergens. Using the sera from 20 patients, human IgE binding of two of the proteins, BbEno1 and BbAld, was shown. Due to the small sample size and because the sera used to screen for allergenicity was not derived form patients with know *B. bassiana* allergies, the lack of reactivity of BbF2 and BbHex does not discount them as potential allergens.

Allergenic fungal enolases have been shown to be highly cross reactive, and it has been reported that IgE cross-reactivity exists between the enolases of at least five fungal

species, extending even to the plant enolase (*Hevea brasiliensis*) (Simon-Nobbe et al., 2000; Wagner et al., 2000). Phylogenetic analysis of available fungal enolase sequences, including those shown to be allergens (and cross-reactive) resulted in no clear distribution pattern. The enolases which have been identified as allergens were distributed equally throughout the phylograms. This could indicate that the shape or function of the enzyme has an effect on the immune response, resulting in a preference towards humeral and IgE pathways. This has been seen with other highly allergenic enzymes (Gough et al., 2003; Gough et al., 2001).

The cross-reactive nature of these enolases increases their importance as causes of allergic reactions. The cross reactive enolases are seen throughout the phylogram and even include cross-reactivity between fungal and non-fungal enolases. This indicates that not only does the enzymatic enolase have a propensity towards humeral immune response but that the allergenic region of the protein is highly conserved even between distant taxa.

Although based on sequence similarity, BbEno1 has been designated as an enolase and BbAld as an aldehyde dehydrogenase, biochemical confirmation is still required. Immunoblot results demonstrated both BbEno1 and BbAld are bound by specific human IgEs and can therefore elicit allergic responses. BbEno1 and BbAld represent the first identification of allergens from the filamentous fungus *B. bassiana*. Future research is aimed at confirming the function of BbEno1 and BbAld, as well as continuing to isolate and identify the epitopes responsible of *B. bassiana* allergenicity.

Table 3-1. PCR Primers

Clone	Primer ID	Primer sequence (5'–3')	Function
BbEno1	11010110i-r	cctcggcgaaggggtcttcgatg	5' RACE
	11010110i-f	atgattgggaggcctggagctacttctaca	3' RACE
	Beab1-f	gaaagacagtccatatggccatcaccaagg	Forward
	Beab1-r	gaattccgtcacgccgcatgtcagcactcc	Reverse
BbF2	11130106i-f	gggctgcgacatcgtacgcccaaa	5' RACE
	11130106i-r	ccggagttggatgcactggcaagct	3' RACE
	Beab2-f	gacatcacaatccatatgaagacaccgagc	Forward
	Beab2-r	gaattcgacaatacatttgcttcatccaccgcactc	Reverse
BbAld	3H05-f	tcaggt tccaggaatgcagcagctttga	5' RACE
	3H05-r	agaaggtcactcttgagctcggtggcaagt	3' RACE
	Beab3-f2	catatgactttgacagtgcagctatctacgcccgct	Forward
	Beab3-r	gaattctgttgatgtcccaagagcttgtctgggc	Reverse
BbHex	5'HexosRace	aacgagggggtggccgcagt	5' RACE
	5'HexosRace2	gcgcgcgtatgcaatgaggtctttaac	5' RACE
	Beab4-f	catatgcgttctcagtcattgtcctctggtttgc	Forward
	Beab4-r	gaattcgaatgacaagtcctacactattgccgctgctcc	Reverse

Modified or inserted base pairs are underline.

Table 3-2. Cloning vectors

Plasmid	Gene	*E. coli* strain	Notes	Cell stock
BbEno1				
pCR2.1-TOPO	cDNA	Top 10	NdeI/EcoRI incorporated	G20a
pCR2.1-TOPO	genomic	Top 10	Sequence confirmed	G13
pET43.1a	cDNA	BL21(DE3)	Sequence confirmed	G21a
pET43.1a	cDNA	BL21(DE3)	pRARE/expression	G23a
BbF2				
pCR2.1-TOPO	cDNA	Top 10	NdeI/EcoRI incorporated	G20b
pCR2.1-TOPO	genomic	Top 10	Sequence confirmed	G14
pET43.1a	cDNA	BL21(DE3)	Sequence confirmed	G21b
pET43.1a	cDNA	BL21(DE3)	pRARE/expression	G22b
BbAld				
pCR2.1-TOPO	cDNA	Top 10	NdeI/EcoRI incorporated	G24
pCR2.1-TOPO	genomic	Top 10	Sequence confirmed	G15
pET43.1a	cDNA	Top 10	Sequence confirmed	G25
pET43.1a	cDNA	BL21(DE3)	pRARE/expression	G26
BbHex				
pCR2.1-TOPO	cDNA	Top 10	NdeI/EcoRI incorporated	G20d
pCR2.1-TOPO	genomic	Top 10	Sequence confirmed	G16
pET43.1a	cDNA	BL21(DE3)	Sequence confirmed	G21d
pET43.1a	cDNA	BL21(DE3)	pRARE/expression	G23d

Table 3-3. Allergens with sequence similarities to *B. bassiana*

Species	Function	Allergen I.D.	Accession number	E* value
BbEno1				
Alternaria alternata	enolase	Alt a 6	U82437	0.0
Cladosporium herbarum	enolase	Cla h 6	X78226	0.0
Aspergillus fumigatus	enolase	Asp f 22w	AF284645	0.0
Neurospora crassa	enolase	-	XM323150	0.0
Penicillium citrinum	enolase	Pen c 22w	AF254643	0.0
BbF2				
Aspergillus fumigatus	major allergen	Asp f 2	AAC59357	-64
Aspergillus nidulans	antigen 1	-	XP659435	-55
Candida albicans	pH regulated antigen	-	AAC00525	-52
Candida albicans	fibrinogen binding mannoprotein	-	AAC49898	-52
BbAld				
Alternaria alternata	aldehyde dehydrogenase	Alt a 10	X78227	0.0
Cladosporium herbarum	aldehyde dehydrogenase	Cla h 10	X78228	0.0
Cladosporium fulvum	aldehyde dehydrogenase	-	AF275347	0.0
Neurospora crassa	aldehyde dehydrogenase	-	XM951769	0.0
Aspergillus nidulans	aldehyde dehydrogenase	-	XM653066	0.0
BbHex				
Metarhizium anisopliae	acetylglucosaminidase	-	DQ000319	0.0
Aspergillus fumigatus	acetylhexosaminidase	-	XM742214	0.0
Aspergillus oryzae	acetylhexosaminidase	-	AB085840	0.0
Penicillium chrysogenum	acetylglucosaminidase	Pen ch 20	AAB34785	-47

* E=Kmn e^-λS Blastx statistical value; E = the chance the match was made in error.(Fitch, 1983).

Table 3-4. Result of RACE PCR

Clone	Gene	Insert	Length	
			Atg–stop	poly a
11190110i	*bbeno1*	original SSH clone	271	-
2110202	*bbeno1*	5' RACE product	907	-
2250210	*bbeno1*	3' RACE product	410	601
	bbeno1	Total sequence	1317	1508
11130106i	*bbf2*	original SSH clone	190	388
2110205	*bbf2*	5' RACE product	736	-
2200211	*bbf2*	3' RACE product	50	217
	bbf2	Total sequence	786	984
3H05	*bbald*	original SSH clone	195	-
2110209	*bbald*	5' RACE product	775	-
2250212	*bbald*	3' RACE product	719	839
	bbald	Total sequence	1494	1618
1H10		original SSH clone	651	787
6170201	*bbhex*	5' RACE product	864	-
7260201	*bbhex*	5' RACE 2nd round	912	-
	bbhex	Total sequence	1959	2095

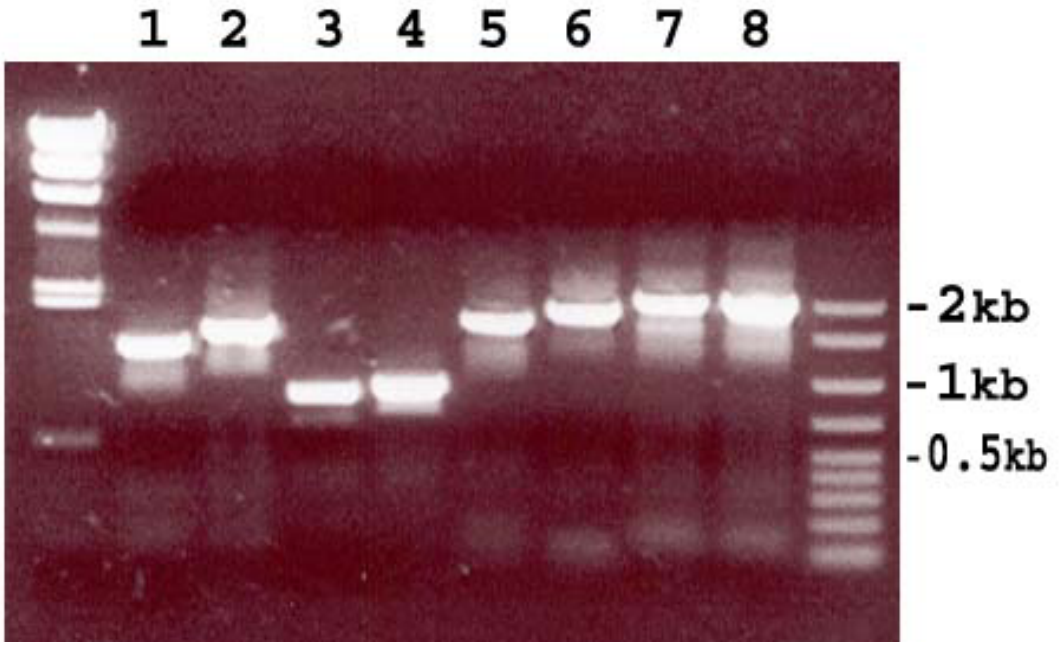

Figure 3-1. cDNA vs genomic: (2)*bbeno1*, cDNA ;(3) *bbeno1*, genomic DNA; (4) *bbf2* cDNA; (5) *bbf2*, genomic DNA; (6) *bbald*, cDNA; (7) *bbald*, genomic DNA; (8) *bbhex*, cDNA; (9) *bbhex*, genomic DNA; (1) lambda DNA, Hind III digest (New England Biolabs, MA), (10) 50–2kb ladder (BioRad, CA).

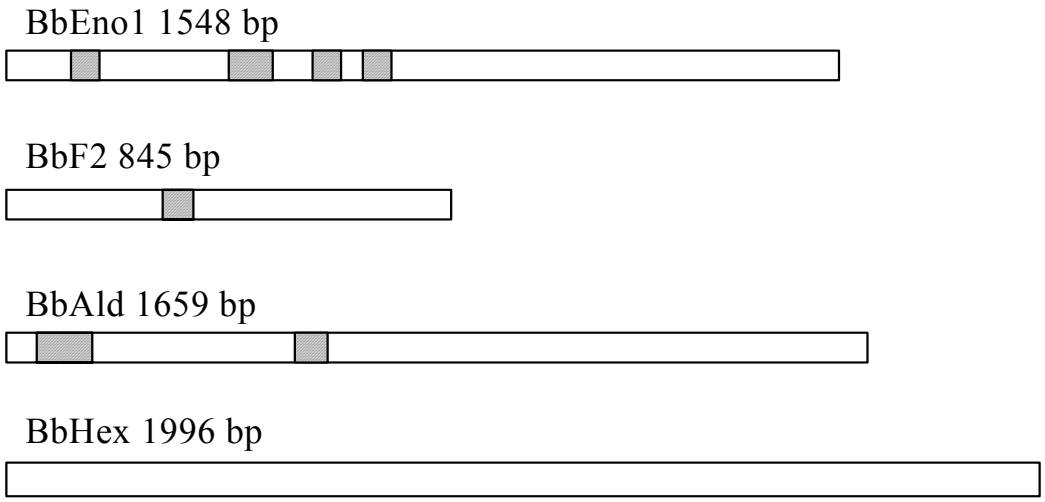

Figure 3-2. Illustration depicting genomic gene sequences of putative *B. bassiana* allergens with relative size and location of introns.

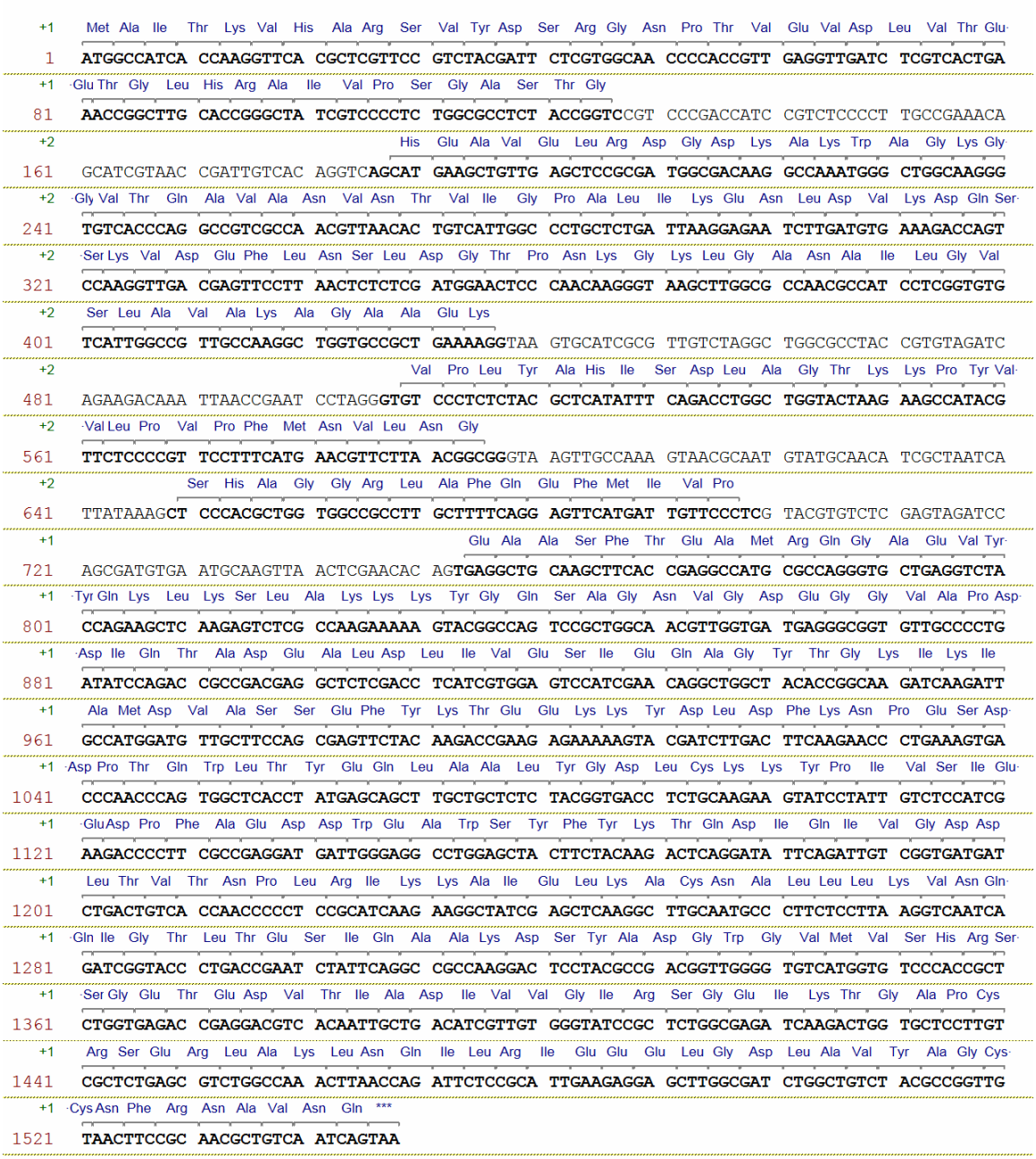

Figure 3-3. Genomic nucleotide sequence and amino acid translation of *bbeno1*.

Figure 3-4. Genomic nucleotide sequence and amino acid translation of *bbf2*.

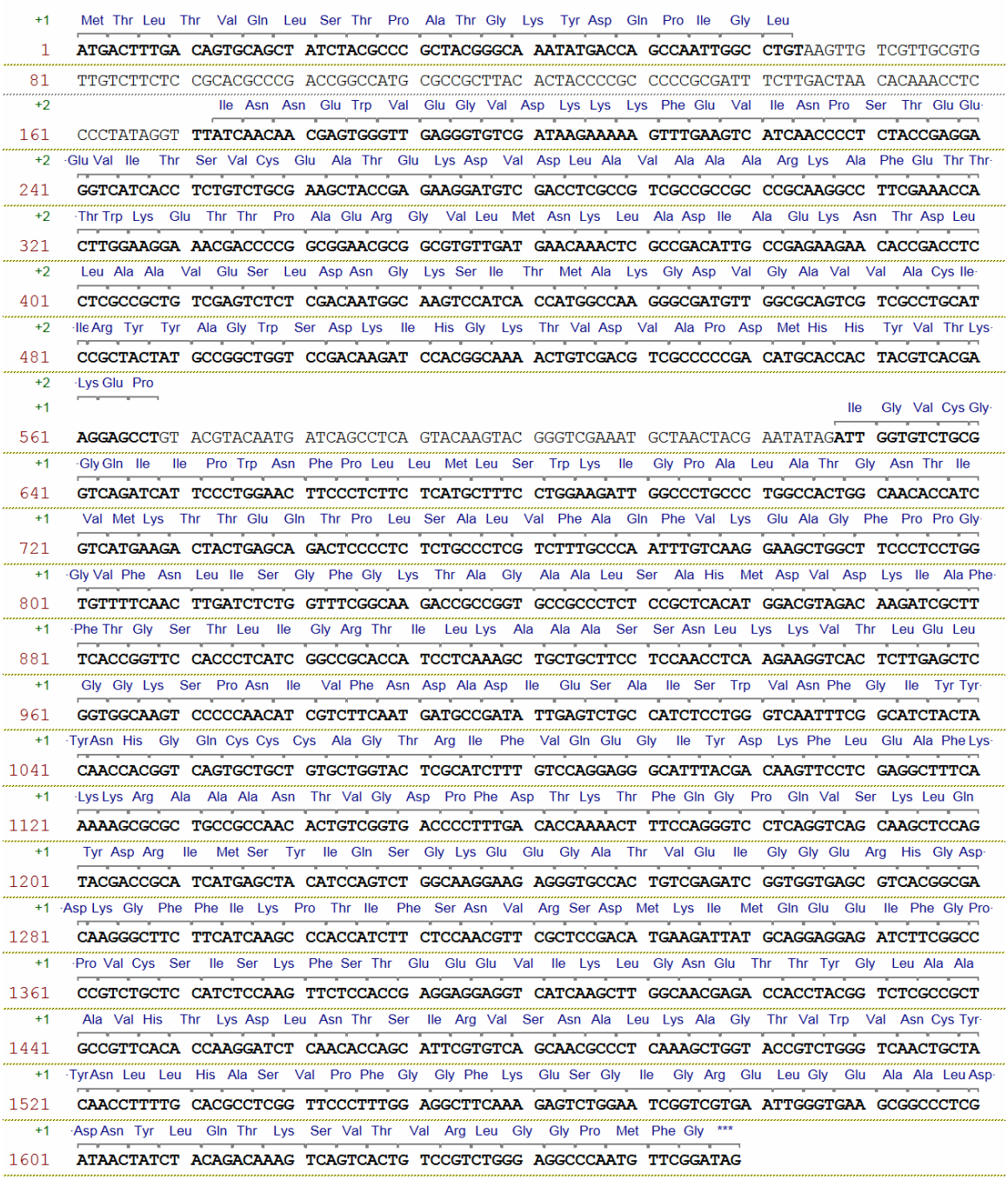

Figure 3-5. Genomic nucleotide sequence and amino acid translation of *bbald*.

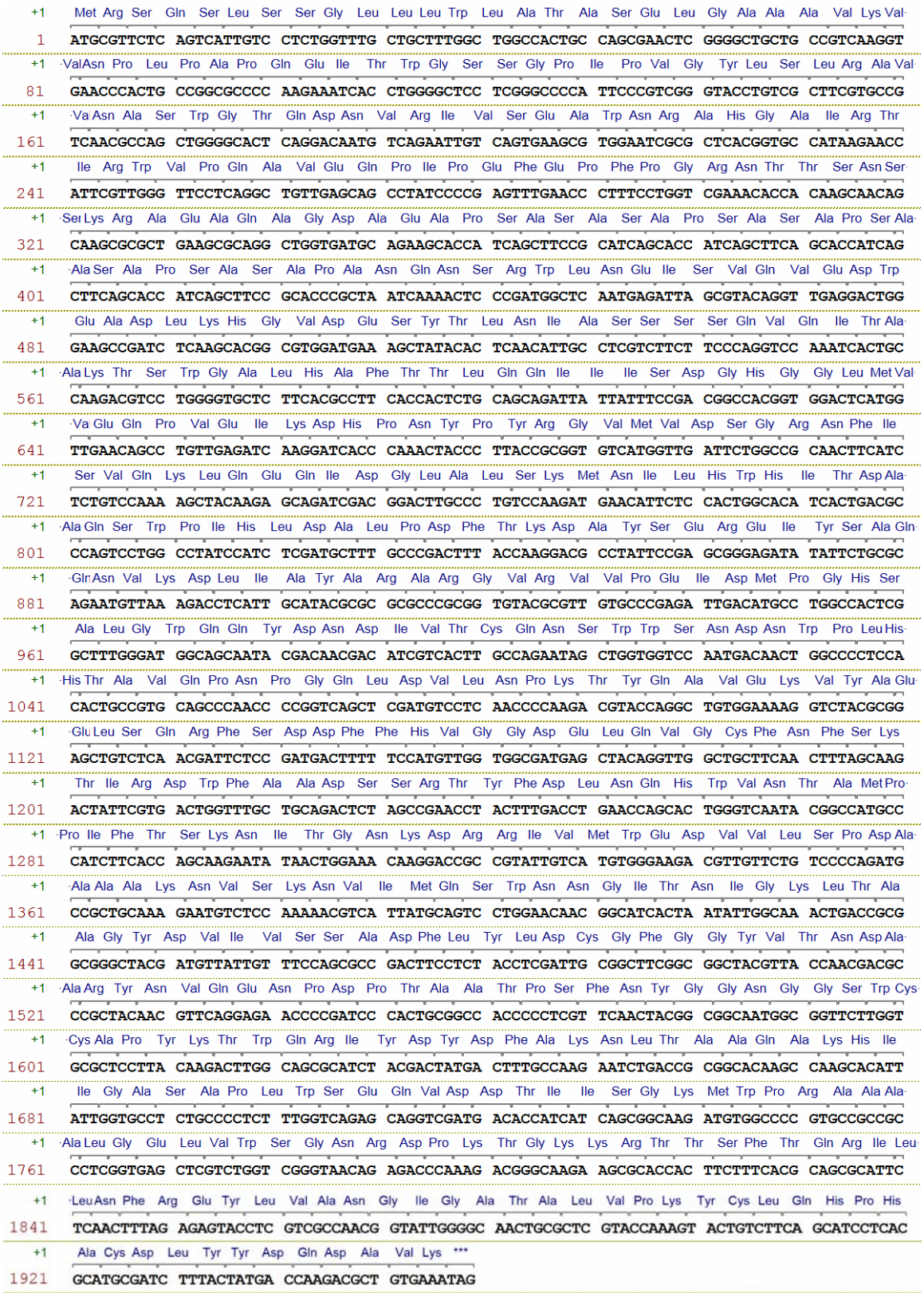

Figure 3-6. Genomic nucleotide and amino acid sequence of *bbhex*.

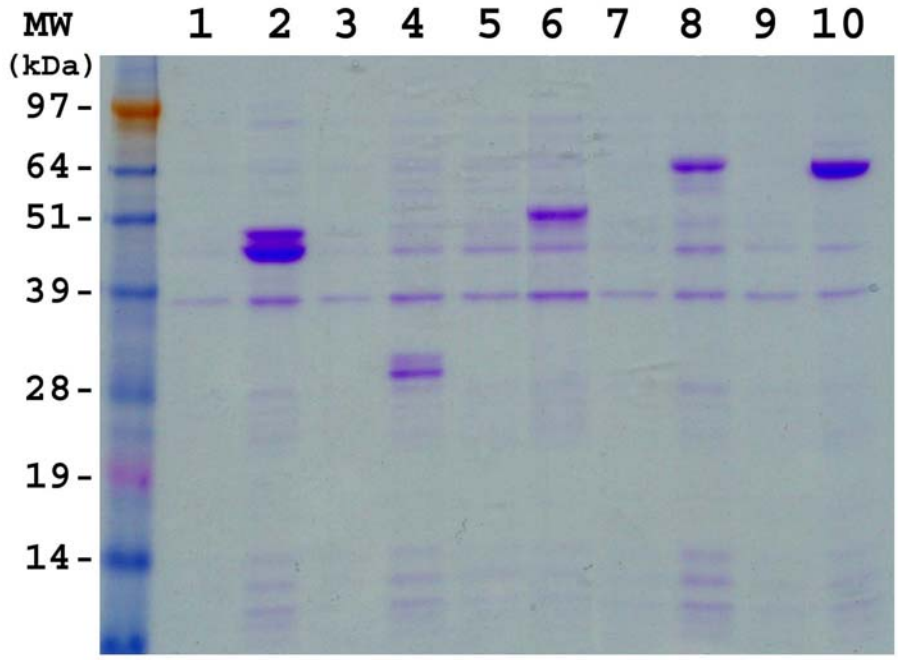

Figure 3-7. SDS-PAGE gel of unfractioned uninduced and induced expression cultures, and stained with Coomasie Blue. Lane (1) BbEno1 uninduced, (2) BbEno1 induced, (3) BbF2 uninduced, (4) BbF2 induced, (5) BbAld uninduced, (6) BbAld induced, (7) BbHex uninduced, (8) BbHex induced, (9) uninduced unmodified pET vector and (10) induced unmodified pET vector.

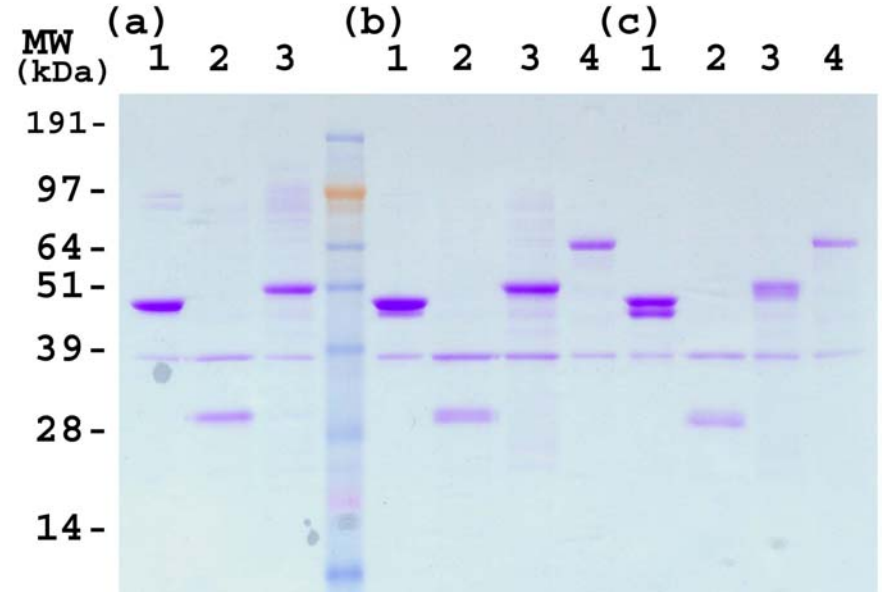

Figure 3-8. Coomasie Blue stained 12% SDS-PAGE gel. Lane (1) BbEno1 pellet fraction, (2) BbF2 pellet fraction, (3) BbAld pellet fraction, and (4) BbHex pellet fraction. Proteins treated with 1x LDS sample buffer and incubated at 95˚C for (a) 1 minute, (b) 5 minutes, and (c) 20 minutes.

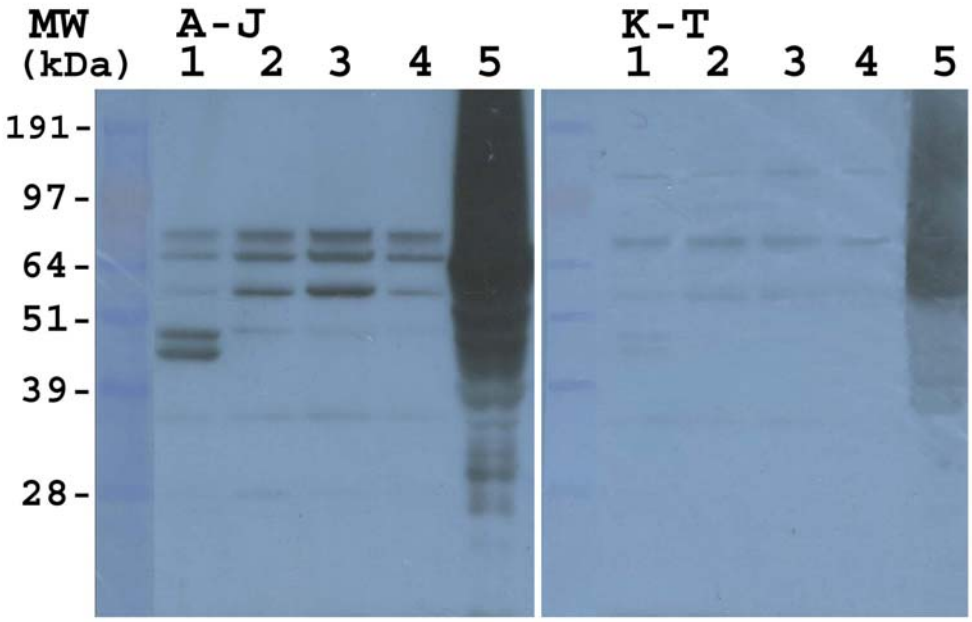

Figure 3-9. Immunoblot: panels designated according to the sera in the pool it was probed with. 10 sera per pool, final concentration of each serum (1:35). Lane (1) BbEno1 induced sample, (2) BbF2 induced sample, (3) BbAld induced sample, (4) BbHex induced sample, and (5) 40 µg crude *B. bassiana* extract.

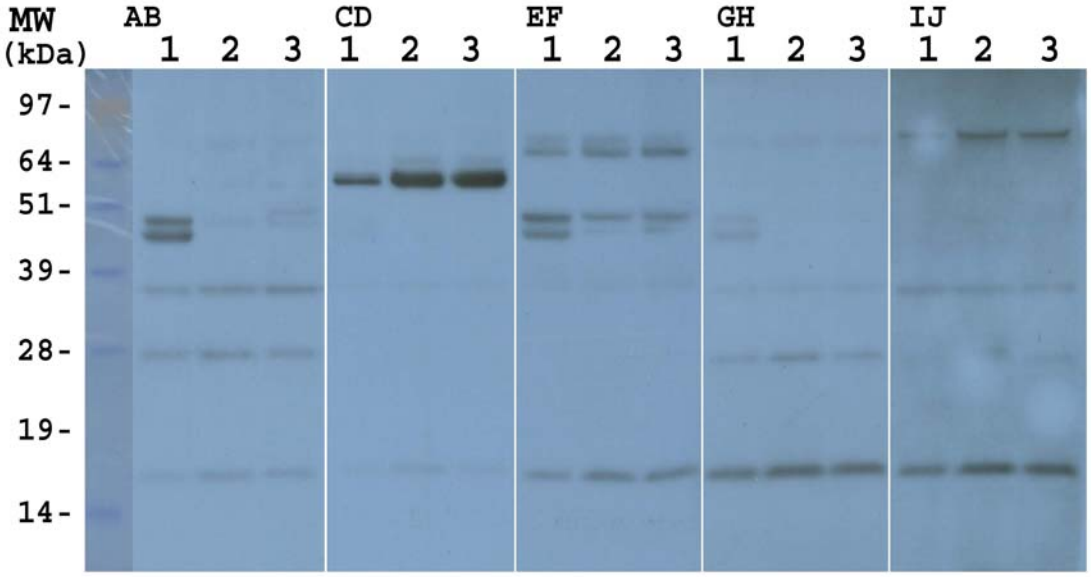

Figure 3-10. Immunoblot panels are designated according to the sera in the pool it was probed with. 2 sera per pool, final concentration of each serum (1:5). Lane 1–3, induced culture (unfractioned) (1) BbEno1 sample (2) BbF2 sample (3) BbAld sample.

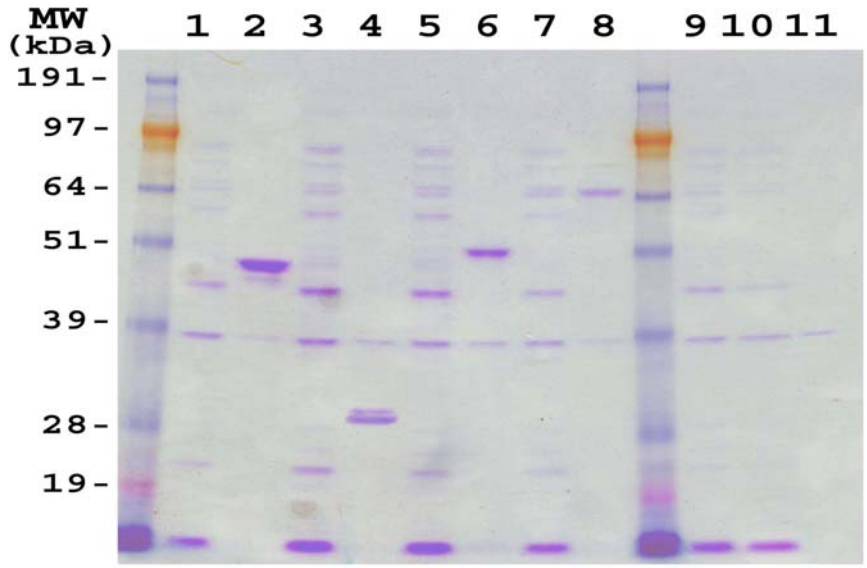

Figure 3-11. 10%SDS-PAGE gel stained with Coomasie. (1) BbEno1 induced soluble fraction; (2) BbEno1 induced pellet fraction; (3) BbF2 induced soluble; (4) BbF2 induced pellet fraction; (5) BbAld induced soluble; (6) BbAld induced pellet fraction; (7) BbHex induced soluble; (8) BbHex induced pellet fraction; (9) BbHex uninduced soluble; (10) BbHex uninduced unfractioned; (11) BbHex uninduced pellet fraction.

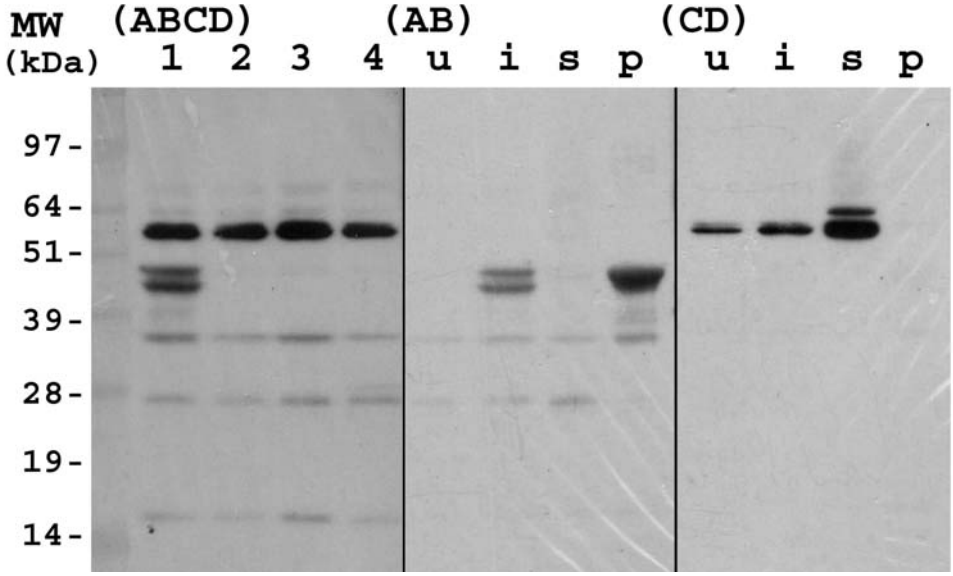

Figure 3-12. Immunoblots probed with pooled sera final dilution of each serum in each pool (1:10). Panals are labeled according to screening pool. Lane (1) BbEno1 induced unfractioned, (2) BbF2 induced unfractioned (3) BbEno1 uninduced unfractioned, (4) BbHex induced unfractioned. (u) BbEno1 uninduced unfractioned, (i) BbEno1 induced unfractioned, (s) BbEno1 induced soluble fraction, (p) BbEno1 induced pellet fraction.

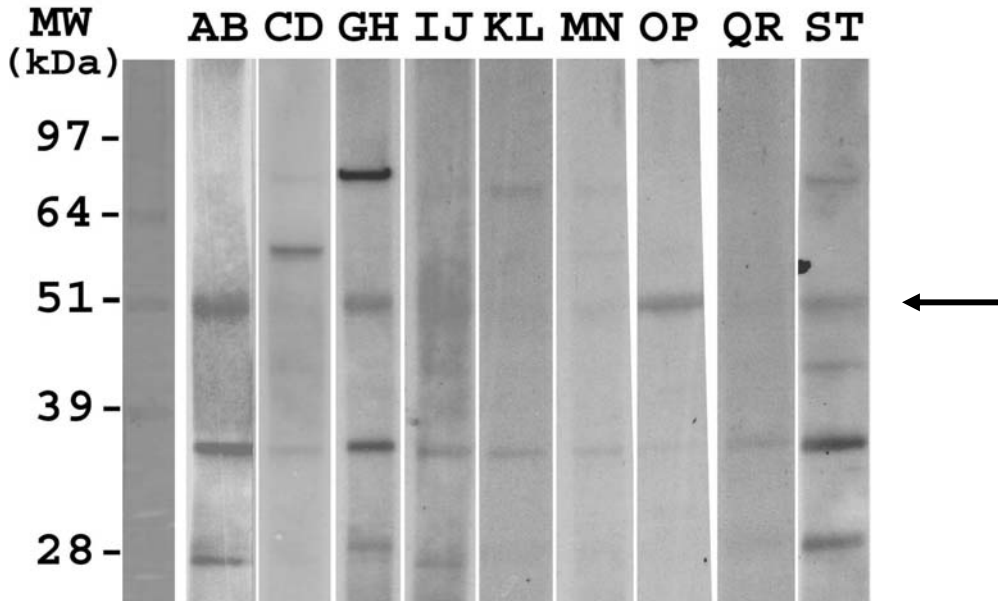

Figure 3-13. Immunoblots of BbAld protein strips probed with 1 mL of sera pool. Each pool contained two sera (final dilution 1:5 each sera) lanes are label according to the sera in the pool it was probed with.

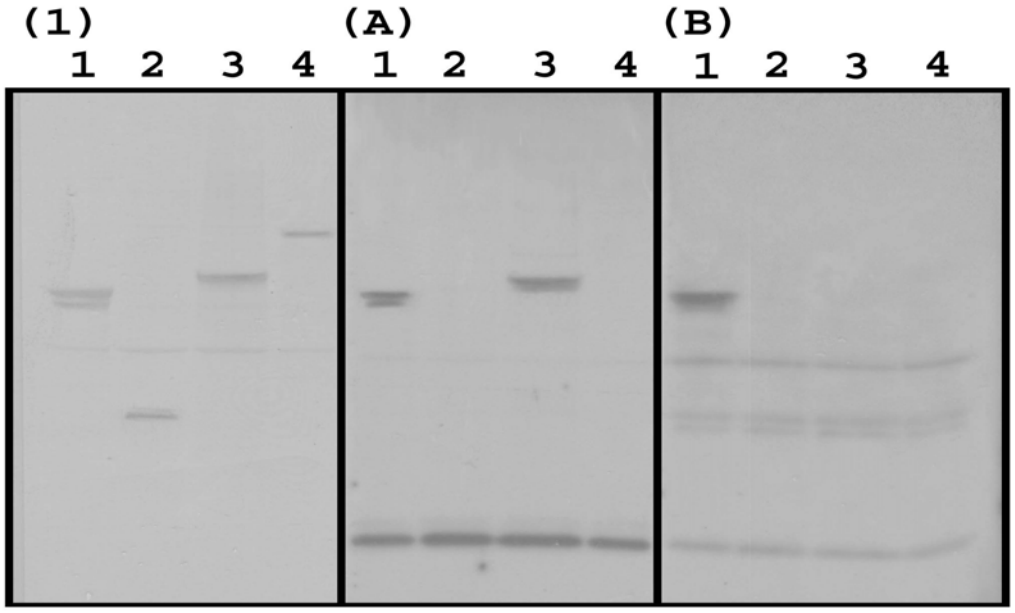

Figure 3-14. Immunoblots of *B. bassiana* proteins (pellet fraction). Lane (1) BbEno1, (2) BbF2, (3) BbAld, and (4) BbHex; panel (1) PVDF membrane stained with Ponceaus S; panel (A) blot treated with serum A (1:5) as primary antibody; panel (B) blot treated with serum B (1:5) as primary antibody.

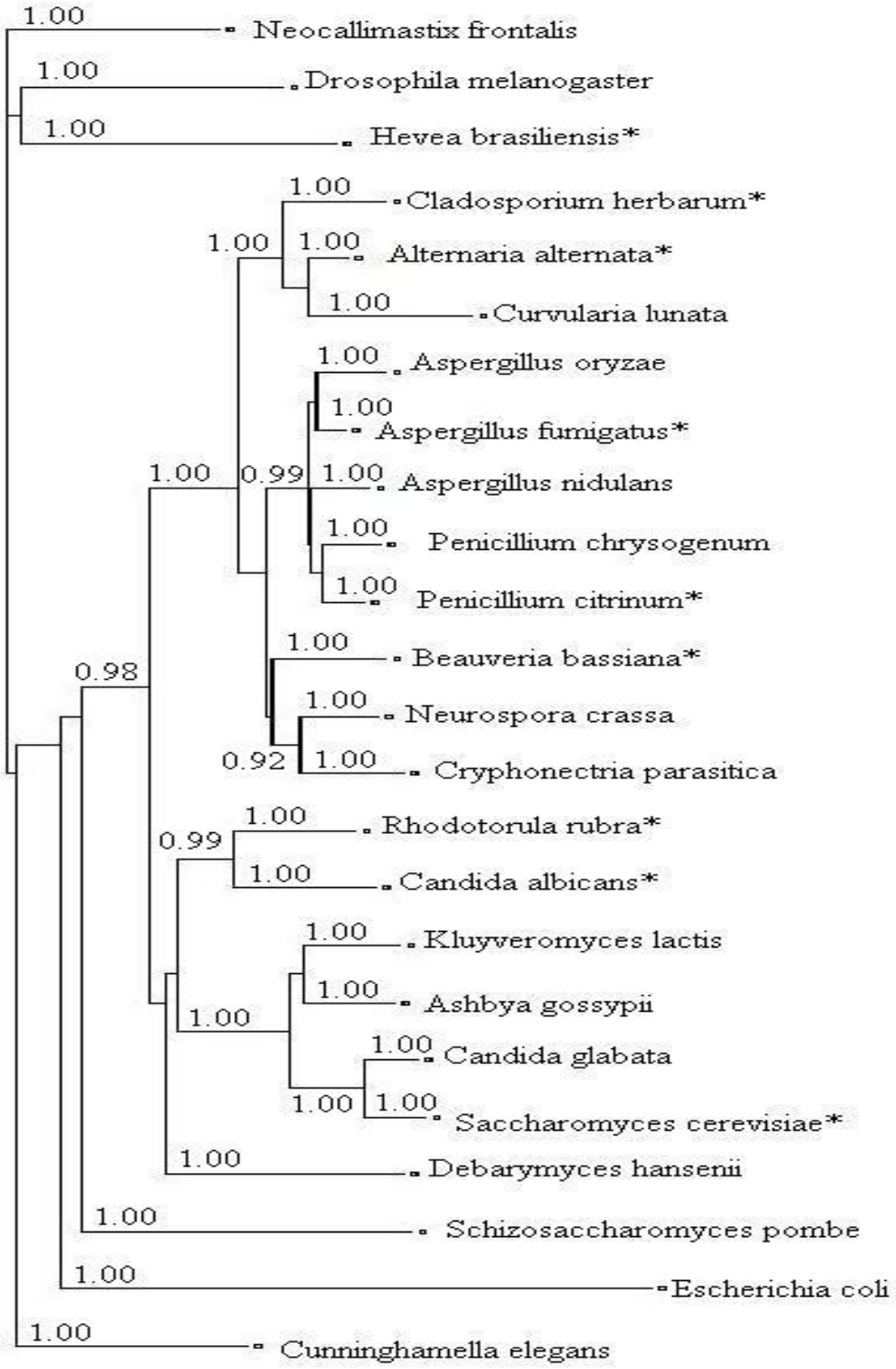

Figure 3-15. Enolase phylogram, numbers at nodes are posterior probabilities values greater than or equal to 90%. Species that produce an enolase known to be allergenic are denoted by an asterisk.

Table 3-5. Enolase accession numbers

Species	Accession number
Alternaria alternata	U82437
Ashbya gossypii	Q756H2
Aspergillus fumigatus	AF284645
Aspergillus nidulans	XM_658258
Aspergillus oryzae	D64113
Beauveria bassiana	DQ767719
Cladosporium herbarum	X78226
Candida albicans enolase	L04943
Candida glabrata	Q6FTW6
Cryphonectria parasitica	Q6RG04
Cunninghamella elegans	O74286
Curvularia lunata	AY034826
Debaryomyces hansenii	Q6BTB1
Drosophila melanogaster	NM_164434
Escherichia. coli	P0A6Q1
Hevea brasiliensis	Q9LEJ0
Kluyveromyces lactis	AJ586240
Neocallimastix frontalis	P42894
Neurospora crassa	XM_323160
Penicillium chrysogenum	AB091508
Penicillium citrinum	AF254643
Rhodotorula rubra	Q870B9
Saccharomyces cerevisiae	J01323
Schizosaccharomyces pombe	P40370

CHAPTER 4
CONCLUSIONS

In industrialized nations, allergic disease is a growing health concern with symptoms ranging from atopic hay fever (sneezing, itching, and coughing) to chronic disease even death. Allergens produced by filamentous fungi contribute to symptoms in all three categories, and therefore pose a human health threat independent of pathogenicity or virulence. Angioedema, hypersensitivity pneumonitis, sinusitis, and asthma are example of serious acute and chronic allergen induced diseases caused by fungi without actual infection.

Beauveria bassiana is an entomopathogenic fungus currently used as a biological pesticide and studied as a potential tool for controlling the spread of insect borne diseases (Geetha and Balaraman, 1999; Haraprasad N, 2001; Scholte et al., 2005; Shah and Pell, 2003). *B. bassiana* was tested and approved for commercial use by the U.S. Environmental Protection Agency after tests showed that *B. bassiana* does not pose a threat of infection to humans or other vertebrates (EPA, 2000). Although not a threat as an infectious disease, *B. bassiana* is a filamentous fungus that may pose a health concern as an allergen; this is especially true for individuals working directly with the fungus in an industrial or agricultural setting, where aerial conidia concentration would be highest.

Human sera and immunoblot analysis were used to study the ability of *B. bassiana* to react with human IgE in order to gauge the validity of the following hypothesis: *B. bassiana* is a filamentous fungus capable of initiating an IgE-mediated hypersensitive response in humans; a response mediated by specific IgEs due to direct sensitivity

developed towards *B. bassiana* allergens, or epitope recognition of a *B. bassiana* antigen(s) by specific IgEs produced in response to another species of fungus (cross-reactivity) (Aukrust and Borch, 1985).

To this point in our study, we revealed that *B. bassiana* produces many IgE reactive proteins, ranging from 12 kDa to >95 kDa, with the most prominent antigens at 35, 42–52, and 60–64. Immunoblots place the allergenic proteins BbEno1 and BbAld in the region of 42–52 kDa and are suspected to be the cause of IgE reactivity in this region. Continued research will concentrate on confirming the role these proteins play in *B. bassiana* hypersensitivity, as well as identifying the remaining major allergenic proteins produced by *B. bassiana*.

Allergenicity of *Beauveria bassiana*

Proteins produced by *B. bassiana* were probed by human sera, and tested for the binding of sera IgEs. Experiments resulted in clear reactivity between extract proteins and human IgE. Reactive proteins bands varied in size and intensity, with the strongest bands at 35, 42–52, and 60–64 kDa. Western blots probed with individual sera confirmed that antibody-antigen interactions are the result of specific recognition of *B. bassiana* proteins by sera IgEs. Although common bands can be seen between individuals, each serum produced a unique banding pattern due to the variation in reactive IgEs. The most common band was located at 35 kDa band, which was present in 6 of the 10 sera showing IgE reactive. Only two sera had the identical reaction to *B. bassiana*, both patients displayed reaction to the 35 kDa protein alone. Of the individual serum tested, 13 came from patients with known fungal allergies; all had tested positive for allergic reactions to at least two other species of fungi. Of these 13 patients, 8 sera tested positive for IgE binding to *B. bassiana* proteins.

To address the issue of cross-reactivity, competitive inhibition experiments showed that *B. bassiana* shared several allergenic epitopes with other common allergenic fungi. Although no single fungus removed all bands, *Alternaria* and *Epicoccum* shared the most allergenic epitopes. No fungus removed the 35 kDa band which may represent direct sensitivity to *B. bassiana*. Skin tests confirm the ability of *B. bassiana* proteins to elicit an IgE specific allergic response.

Characterization of Allergens

Screening of EST and SSH libraries (Holder, 2005), revealed proteins with sequence similarity to major fungal allergens; the proteins were cloned and designated BbEno1, BbF2, BbAld, and BbHex. Of the four, BbEno1 was of particular interest due to its sequence similarities to a highly cross-reactive group of fungal enolases. Of the twenty fungal enolase sequences found in the NCBI protein data base, seven have been identified as major allergens. Fungal enolase has been called a pan-allergen, since cross reactivity has been shown to exist between epitopes shared by at least five allergenic fungal enolases, cross-reactivity has also been seen between fungal and plant enolases (Breitenbach and Simon-Nobbe, 2002; Simon-Nobbe et al., 2000).

Phylogenetic comparisons of enolase sequences show that allergenic and cross-reactive epitopes are not limited to a specific group of fungi, but are distributed throughout the cladogram and includes *Hevea brasiliensis* (non-fungal enolase). For this reason, it is likely that more of the identified enolases will prove to be allergens once tested.

The putative *B. bassiana* enolase, BbEno1, was tested for allergenicity by probing western blots with human serum. Sera came from patients with known fungal allergens, and blots confirmed that BbEno1 is recognized and bound by specific IgE(s). BbEno1

was tested with several different sera pools showing the IgE binding is specific to the BbEno1 protein and that the reaction occurs in a significant percent of the patient sera tested. Due to the conserved nature of fungal enolases it is likely that BbEno1 will prove to be cross-reactive with IgEs from other allergenic enolases.

BbAld was also shown by Immunoblot analysis using human sera to be capable of initiating an allergic response by binding sera IgEs. Although not as numerous as fungal enolase, aldehyde dehydrogenases are include in the list of major and minor fungal allergens. *Alternaria alternata* and *Cladosporium herbarium* are two fungi that posses aldehyde dehydrogenase that are not only allergenic but also believed to be cross-reactive (Kurup and Banerjee, 2000).

Future Experiments

We have shown that *B. bassiana* produces many proteins capable of initiation a human allergic response either by cross-reactivity or by direct developed sensitivity to *B. bassiana* antigens. Although we have isolated and identified two allergenic proteins, continued work is needed to identify the remaining allergens, as well as further characterization of BbEno1 and BbAld. Future research will concentrate on three areas; (1) the continued identification of allergens; (2) the functional and biochemical characterization of allergens; (3) development of hypoallergenic strains.

Identifying the major allergenic proteins of *B. bassiana* is the primary goal of future research. It is believed that BbEno1 and BbAld are responsible, at least in part, for the high reactive 42–52 kDa region seen in immunoblot assays. Competitive inhibition blots using purified BbEno1 and BbAld can be performed to confirm or identify the role these proteins play in the allergenicity of this region. Identification of the remaining

major bands at 60–64 and especially the 35 kDa is important for understanding the allergenicity of this fungus.

Once identified as allergenic, steps will to be taken to confirm the identity of the protein. By sequence similarity BbEno1 has been designated to be and enolase and BbAld to be aldehyde dehydrogenase. Biochemical function and/or properties of BbEno1, BbAld, and all other *B. bassiana* allergens that are identified, can be confirmation by enzyme assay. Northern blots analysis can be utilized to understand production and regulation of the identified allergens.

The identification, isolation, and characterization, of the major *B. bassiana* allergens are preparatory to the production of knockout strains, which will be used to study the effect or the importance of the proteins in fungal metabolism and virulence. If an identified allergen carries out a redundant function then its removal may not affect its virulence. If a knockout strains result in the loss or significant decrease in function, then restoration of function may be obtained by complementation with non-allergenic forms of the enzyme. *B. bassiana* has great potential in commercial and agricultural pest management as well as insect borne disease control; the production and use of hypoallergenic strains of *B. bassiana* could reduce the potential threat of causing acute or chronic allergic disease.

APPENDIX
ADDITIONAL FIGURES AND TABLES

Table A-1. Taxonomy of *Beauveria bassiana*

	Holomorph	Anamorph
Kingdom	Fungi	
Phylum	Ascomycotina	
Subphylum	Pezizomycotina	Deuteromycota
Class	Sordariomycetes	Hyphomycetes
Subclass	Hypocreomycetidae	
Order	Hypocreales	Moniliales
Family	Clavicipitaceae	
Genus	Cordyceps	Beauveria
Species	bassiana	bassiana

Table A-2. Molecular properties of *B. bassiana* genes

Gene product	MW (kDa)	Putative Function	Intron number	Gene length Gen.	Gene length cDNA	# AA	pI
BbEno1	47.4	Enolase	4	1548	1317	438	5.07
BbF2	28.6	Unknown	1	845	786	261	7.64
BbAld	53.9	Aldehyde dehydrogenase	2	1659	1494	497	5.99
BbHex	72	Hexos-aminidase	0	1959	1959	652	5.56

```
BbEno1    MAITKVHARSVYDSRGNPTVEVDLVTETGLHRAIVPSGASTGQHEAVELRDGDKAKWAGK 60
Aspf22w   MPISKIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEAHELRDGDKTQWGGK 60
alta5     MTITKIHARSVYDSRGNPTVEVDIVTETGLHRAIVPSGASTGSHEACELRDGDKSKWGGK 60
Clah6     MPISKIHSRYVYDSRGNPTVEVDIVTETGLHRAIVPSVASTGSHEACELRDGDKSKWAGK 60
          *.*:*:*:* **************:************** ****.*** *******::*.**

BbEno1    GVTQAVANVNTVIGPALIKENLDVKDQSKVDEFLNSLDGTPNKGKLGANAILGVSLAVAK 120
Aspf22w   GVLKAVKNVNETIGPALIKENIDVKDQSKVDEFLNKLDGTANKSNLGANAILGVSLAVAK 120
alta5     GVTKAVANVNDTIAPALIKEKLDVKDQSAVDAFLNKLDGTTNKTNLGANAILGVSMAIAK 120
Clah6     GVTKAVANVNEIIAPALIKENLDVKDQAAVDAFLNKLDGTTNKTKIGANAILGVSMAVAK 120
          ** :** ***  *.******::*****: ** ***.****.** ::**********:*:**

BbEno1    AGAAEKGVPLYAHISDLAGTKKPYVLPVPFMNVLNGGSHAGGRLAFQEFMIVPSEAASFT 180
Aspf22w   AGAAEKGVPLYAHISDLAGTKKPYVLPVPFQNVLNGGSHAGGRLAFQEFMIVPDSAPSFS 180
alta5     AAAAEKGVPLYAHISDLAGTKKPYVLPVPFQNVLNGGSHAGGRLAFQEFMIVPCEAPTFS 180
Clah6     AAAAEKRVPLYAHISDLSGTKKPFVLPVPFMNVVNGGSHAGGRLAFQEFMIVPSGAPSFT 180
          *.**** **********:*****:****** **:******************  *.:*:

BbEno1    EAMRQGAEVYQKLKSLAKKKYGQSAGNVGDEGGVAPDIQTADEALDLIVESIEQAGYTGK 240
Aspf22w   EALRQGAEVYQKLKALAKKKYGQSAGNVGDEGGVAPDIQTAEEALDLITEAIEQAGYTGK 240
alta5     EAMRQGAEVYQKLKALAKKTYGQSAGNVGDEGGVAPDIQTAEEALDLITKAIEEAGYTGK 240
Clah6     EAMRQGAEVYQKLKSLTKKRYGQSAGNVGDEGGVAPDIQTAEEALDLITDAIEEAGYTGQ 240
          **:***********:*:** ********************:******..:**:*****:

BbEno1    IKIAMDVASSEFYKTEEKKYDLDFKNPESDPTQWLTYEQLAALYGDLCKKYPIVSIEDPF 300
Aspf22w   IKIAMDVASSEFYKADVKKYDLDFKNPESDPSKWLTYEQLADLYKSLAAKYPIVSIEDPF 300
alta5     IKIAMDVASSEFYKADEKKYDLDFKNPDSDKSKWLTYEQLAEMYKSLAEKYPIVSIEDPF 300
Clah6     IKIAMDVASSEFYKADEKKYDLDFKNPDSDKSKWITYEQLADQYNELAAKYPIVSIEDPF 300
          ***************:: **********:** ::*:******  * .*. ***********

BbEno1    AEDDWEAWSYFYKT--QDIQIVGDDLTVTNPLRIKKAIELKACNALLLKVNQIGTLTESI 358
Aspf22w   AEDDWEAWSYFYKT--SDFQIVGDDLTVTNPGRIKKAIELKSCNALLLKVNQIGTLTESI 358
alta5     AEDDWEAWSYFFKTYDG--QIVGDDLTVTNPEFIKKAIELKSCNALLLKVNQIGTITEAI 358
Clah6     AEDDWEAWSYFYKTSGSDFQIVGDDLTVTNPEFIKKAIETKACNALLLKVNQIGTITEAI 360
          ***********:**     ************  ****** *:**************:**:*

BbEno1    QAAKDSYADGWGVMVSHRSGETEDVTIADIVVGIRSGEIKTGAPCRSERLAKLNQILRIE 418
Aspf22w   QAAKDSYADNWGVMVSHRSGETEDVTIADIAVGLRSGQIKTGAPCRSERLAKLNQILRIE 418
alta5     QAAKDAFGAGWGVMVSHRSGETEDVTIADIVVGLRSGQIKTGAPARSERLAKLNQILRIE 418
Clah6     NAAKDSFAAGWGVMVSHRSGETEDVTIADIVVGLRAGQIKTGAPARSERLAKLNQILRIE 420
          :****::. .********************.**:*:*:******.***************

BbEno1    EELGDLAVYAGCNFRNAVNQSTP 441
Aspf22w   EELGENAVYAGSKFRTAVNL--- 438
alta5     EELGDNAVYAGNNFRTAVNL--- 438
Clah6     EELGDKRLYAGDNFRTAINL--- 440
          ****:  :*** :**.*:*
```

Figure A-1. Clastalw alignment of BbEno1 and the allergenic enolases from, *Alternaria alternata* (alt a 6), *Cladosporium herbarum* (Cla h 6), and *Aspergillus fumigatus* (asp f 22w).

```
Clah3    -MTSVQLETPHSGKYEQPTGLFINNEFVKGQEGKTFDVINPSDESVITQVHEATEKDVDI 59
Alta10   -MTSVKLSTPQTGEFEQPTGLFINNEFVKAVDGKTFDVINPSTEEVICSVQEATEKDVDI 59
BbAld    MTLTVQLSTPATGKYDQPIGLFINNEWVEGVDKKKFEVINPSTEEVITSVCEATEKDVDL 60
            :*:*.** :*:::** *******:*:. : *.*:***** *.** .* ********:

Clah3    AVAAARKAFEGSWRQETPENRGKLLNNLANLFEKNIDLLAAVESLDNGKAISMAKG-DIS 118
Alta10   AVAAARKAFNGPWRKETPENRGKLLNKLADLFEKNADLIAAVEALDNGKAFSMAKNVDVP 119
BbAld    AVAAARKAFETTWKETTPAERGVLMNKLADIAEKNTDLLAAVESLDNGKSITMAKG-DVG 119
         *********: .*:: ** :** *:*:**:: *** **:****:*****:::***. *:

Clah3    MCVGCLRYYGGWADKITGKVIDTTPDTFNYVKKEPIGVCGQIIPWNFPLLMWAWKIGPAI 178
Alta10   AAAGCLRYYGGWADKIEGKVVDTAPDSFNYIRKEPIGVCGQIIPWNFPILMWSWKIGPAI 179
BbAld    AVVACIRYYAGWSDKIHGKTVDVAPDMHHYVTKEPIGVCGQIIPWNFPLLMLSWKIGPAL 179
           ..*:***.**:*** **.:*.:** .:*: ****************:** :******:

Clah3    ACGNTVVLKTAEQTPLGGLVAASLVKEAGFPPGVINVISGFGKVAGAALSSHMDVDKVAF 238
Alta10   ATGNTVVLKTAEQTPLSAYIACKLIQEAGFPPGVINVITGFGKIAGAAMSAHMDIDKIAF 239
BbAld    ATGNTIVMKTTEQTPLSALVFAQFVKEAGFPPGVFNLISGFGKTAGAALSAHMDVDKIAF 239
         * ***:*:**:*****.. : ..:::********:*:*:**** ****:*:***:**:**

Clah3    TGSTVVGRTILKAAASSNLKKVTLELGGKSPNIVFEDADIDNAISWVNFGIFFNHGQCCC 298
Alta10   TGSTVVGRQIMKSAAGSNLKKVTLELGGKSPNIVFADADLDEAIHWVNFGIYFNHGQACC 299
BbAld    TGSTLIGRTILKAAASSNLKKVTLELGGKSPNIVFNDADIESAISWVNFGIYYNHGQCCC 299
         ****::** *:*:**.****************** ***::.** ******::****.**

Clah3    AGSRVYVQESIYDKFVQKFKERAQKNVVGDPFAADTFQGPQVSKVQFDRIMEYIQAGKDA 358
Alta10   AGSRIYVQEEIYDKFIQRFKERAAQNAVGDPFAADTFQGPQVSQLQFDRIMGYIEEGKKS 359
BbAld    AGTRIFVQEGIYDKFLEAFKKRAAANTVGDPFDTKTFQGPQVSKLQYDRIMSYIQSGKEE 359
         **:*::*** *****:: **:**  *.***** :.********::*:**** **: **.

Clah3    GATVETGGKRKGDKGYFIEPTIFSNVTEDMKIVKEEIFGPVCSIAKFKTKEDAIKLGNAS 418
Alta10   GATIETGGNRKGDKGYFIEPTIFSNVTEDMKIQQEEIFGPVCTISKFKTKADVIKIGNNT 419
BbAld    GATVEIGGERHGDKGFFIKPTIFSNVRSDMKIMQEEIFGPVCSISKFSTEEEVIKLGNET 419
         ***:* **:*:****:**:******* .**** :********:*:**.*: :.**:** :

Clah3    TYGLAAAVHTKNLNTAIEVSNALKAGTVWVNTYNTLHHQMPFGGYKESGIGRELGEDALA 478
Alta10   TYGLAAAVHTSNLTTAIEVANALRAGTVWVNSYNTLHWQLPFGGYKESGIGRELGEAALD 479
BbAld    TYGLAAAVHTKDLNTSIRVSNALKAGTVWVNCYNLLHASVPFGGFKESGIGRELGEAALD 479
         **********.:*.*:*.*:***:******* ** ** .:****:*********** **

Clah3    NYTQTKTVSIRLGDALFG 496
Alta10   NYIQTKTVSIRLGDVLFG 497
BbAld    NYLQTKSVTVRLGGPMFG 497
         ** ***:*::***. :**
```

Figure A-2. Clastalw alignment of BbAld and the allergenic Aldehyde dehydrogenase from *Alternaria alternata* (alt a 10), and *Cladosporium herbarum* (Cla h 3).

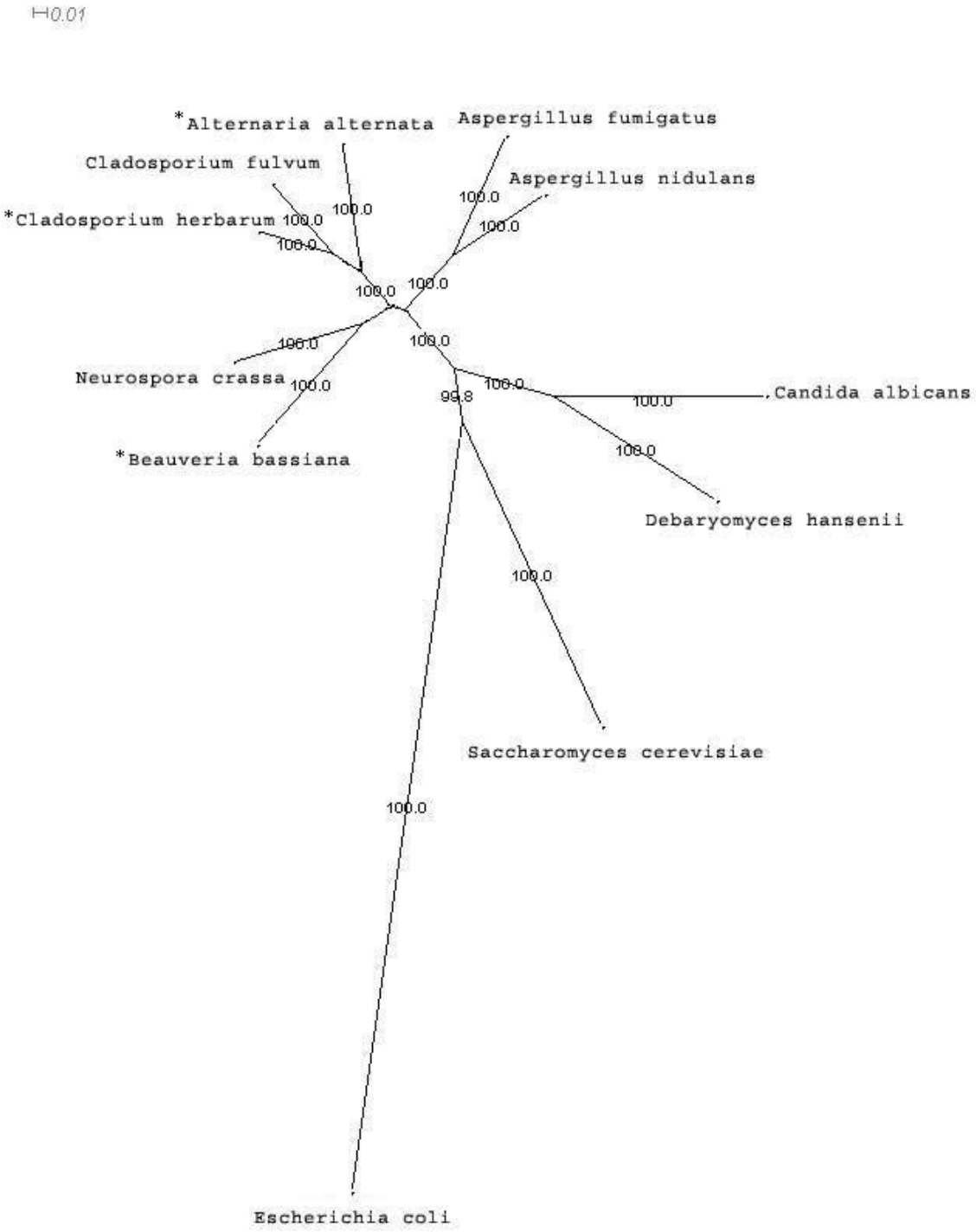

Figure A-3. Aldehyde dehydrogenase phylogram, numbers at nodes are posterior probability values. Species that produce an aldehyde dehydrogenase, known to be allergenic are denoted by an asterisk.

Table A-3. Accession numbers

Species	Accession number	
	Enolase	Aldehyde dehydrogenase
Alternaria alternata	U82437	X78227
Ashbya gossypii	Q756H2	-
Aspergillus fumigatus	AF284645	745933
Aspergillus nidulans	XM_658258	XM_653066
Aspergillus oryzae	D64113	-
Beauveria bassiana	DQ767719	DQ767721
Cladosporium fulvum	-	AF275347
Cladosporium herbarum	X78226	X78228
Candida albicans	L04943	XM_710254
Candida glabrata	Q6FTW6	-
Cryphonectria parasitica	Q6RG04	-
Cunninghamella elegans	O74286	-
Curvularia lunata	AY034826	-
Debaryomyces hansenii	Q6BTB1	XM_461708
Drosophila melanogaster	NM_164434	-
Escherichia. coli	P0A6Q1	P0A9Q7
Hevea brasiliensis	Q9LEJ0	-
Kluyveromyces lactis	AJ586240	-
Neocallimastix frontalis	P42894	-
Neurospora crassa	XM_323160	3873009
Penicillium chrysogenum	AB091508	-
Penicillium citrinum	AF254643	-
Rhodotorula rubra	Q870B9	-
Saccharomyces cerevisiae	J01323	P47771
Schizosaccharomyces pombe	P40370	-

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BIOGRAPHICAL SKETCH

Greg Westwood was born in Texas in 1975, to John and Nate Westwood. As is common for individuals raised in a military home, Greg attended grade schools in Germany, Virginia, and Kansas, before moving to Puyallup, Washington, where he attended Furrucci Junior High School for grades 7–9. High school began at West Springfield High, in Springfield Virginia, where Greg spent all of his tenth-grade year and half of his eleventh before moving to El Paso Texas to finish off his degree. Greg received his diploma from Austin High School in 1994, due in large part to the kindness and understanding of Principle Yturralde.

Greg immediately moved to St. George Utah, where he attending Dixie community college. After a couple of years of hard work and encouragement from his wife, Greg transferred to Southern Utah University in Cedar City. It was at SUU that Greg found his passion for microbiology and began research under the direction of Microbiology professor Dr. Ronald Martin. Greg Westwood received his Bachelor of Science degree from the Southern Utah University in May 2000 and began his graduate education at the University of Florida In August of that same year. Greg received his Ph.D. in August 2006.