

Aspergillus flavus infections in *Galleria mellonella*:
a pathogen-host model system for the study of emerging diseases

by

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ABSTRACT

To study emerging diseases, I employed a model pathogen-host system involving infections of insect larvae with the opportunistic fungus *Aspergillus flavus*, providing insight into three mechanisms of pathogen evolution namely de novo mutation, genome decay, and virulence factor acquisition. In Chapter 2 as a foundational experiment, *A. flavus* was serially propagated through insects to study the evolution of an opportunistic pathogen during repeated exposure to a single host. While *A. flavus* displayed de novo phenotypic alterations, namely decreased saprobic capacity, analysis of genotypic variation in Chapter 3 signified a host-imposed bottleneck on the pathogen population, emphasizing the host's role in shaping pathogen population structure. Described in Chapter 4, the serial passage scheme enabled the isolation of an *A. flavus* cysteine/methionine auxotroph with characteristics reminiscent of an obligate insect pathogen, suggesting that lost biosynthetic capacity may restrict host range based on nutrient availability and provide selection pressure for further evolution. As outlined in Chapter 6, cysteine/methionine auxotrophy had the pleiotrophic effect of increasing virulence factor production, affording the slow-growing auxotroph with a modified pathogenic strategy such that virulence was not reduced. Moreover in Chapter 7, transformation with a virulence factor from a facultative insect pathogen failed to increase virulence, demonstrating the necessity of an appropriate genetic background for virulence factor acquisition to instigate pathogen evolution.

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“[Jesus Christ] is the image of the invisible God, the first born over all creation. For by him all things were created: things in heaven and on earth, visible and invisible, whether thrones or powers or rulers or authorities; all things were created by him and for him. He is before all things, and in him all things hold together.” Colossians 1: 15-17 (NIV)

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Chapter I: Introduction

Emerging infectious diseases and the justification of the use of *Aspergillus flavus* infections in *Galleria mellonella* larvae as a model pathogen-host system for the study of pathogen evolution

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INTRODUCTION

Throughout the centuries, various pandemics of infectious diseases have devastated humanity. Although the advances of modern science and medicine have quelled many of these diseases, a number of novel emerging human pathogens have been identified (Taylor et al. 2001). Quashing an emerging disease before it causes wide-spread infection is surely favorable to attempting to control a pathogen during a full-blown pandemic. However, impeding the emergence of novel human diseases requires an understanding of the factors that cause their surfacing. Although a variety of factors are thought to contribute to the emergence of novel diseases, including a number of environmental or ecological changes, genetic changes ensuing in pathogen evolution are crucial (Morens et al. 2004). Simply speaking, pathogen evolution involves the adaptation of a microbe to the environment of its host. Increases in multiplication and transmission rates are indicative of microbial adaptation but also concomitantly increase pathogen virulence. Thus, pathogens theoretically evolve from saprobes to opportunists to host-adapted, virulent facultative or obligate pathogens (Scheffer 1991). Current studies on the mechanisms of pathogen evolution typically analyze the genomes of known human pathogens in order to uncover their evolutionary histories. These studies indicate that horizontal virulence gene acquisition, de novo mutation and selection resulting from association, and genome decay are three elements associated with pathogen evolution (Wren 2003).

Current theories on pathogen evolution would benefit from a model pathogen-host system in which experimental evolution and manipulation could attempt to recreate the evolution of a novel, virulent pathogen from an opportunist. *Aspergillus flavus* infections in *Galleria mellonella* larvae represent an ideal model pathogen-host system. *A. flavus* is an opportunistic pathogen, infecting insects in addition to a number of other hosts but with low virulence (Yu et al. 2005). Its pathogenic cycle is typical of entomopathogens, and thus is well-defined (Kumar et al. 2004), and it is readily subjected to genetic analysis and manipulation. Insects such as *G. mellonella* larvae are excellent model hosts because they do not pose the ethical issues associated

with the use of mammalian hosts. Also, the commonalities between the insect and mammalian innate immune systems result in a similar response to a number of tested opportunistic pathogens (Brennan et al. 2002; Cotter et al. 2000; Heine & Lien, 2003; Hoffmann et al. 1999; Jander et al. 2000; Medzhitov 2001; Medzhitov & Janeway, 1997), indicating that insects provide a similar environment and analogous selection pressures for pathogens as mammalian hosts. Furthermore, the pathogenic infection cycle in insects and mammals progresses through the same stages, such as host invasion, multiplication and dissemination, immune system avoidance, and transmission. For these reasons, any insights gained concerning the mechanisms and essential steps of pathogen evolution can be applied to the emergence of novel human diseases. *A. flavus* infections in *G. mellonella* larvae represent a superb model system for the study of emerging diseases.

THEORIES AND GENETIC MECHANISMS OF THE EVOLUTION OF NOVEL

PATHOGENS

The threat and prevalence of emerging diseases

Historically, infectious diseases have posed a tremendous threat to human health, life expectancy, and quality of life. In addition to endemic diseases that undoubtedly have claimed a large percentage of lives, various epidemics and pandemics have frequently ravaged humanity, periodically decimating large proportions of the human population. With the advent of the germ theory of disease due to the work of Louis Pasteur and Robert Koch in the mid-1800s, tremendous advances were made in the discovery, control, and treatment of the infectious agents that caused the diseases. Notably, the discovery of penicillin by Alexander Fleming in 1929 and its subsequent mass production proved to be a formidable weapon in the fight against life-claiming bacterial infections. These advancements were so promising that in 1967, the U.S. Surgeon General declared that “the war against infectious diseases has been won” (Morens et al. 2004).

Nevertheless 40 years later, approximately one quarter (15 million) of the 57 million deaths each year are directly caused by infectious diseases (Morens et al. 2004). In fact, a recent catalog of infectious agents identifies 1415 species that are designated as human pathogens. Of these, 217 are viruses and prions, 538 are bacteria and rickettsia, 307 are fungi, 66 are protozoa, and 287 are helminths (Taylor et al. 2001). Clearly, the battle against infectious microorganisms has not been won. However, in addition to the many established and clearly defined human pathogens, there are a number of pathogens (175 of 1415) considered to be emerging human pathogens (Taylor et al. 2001). Emerging pathogens are defined as “diseases of infectious origin whose incidence in humans has increased within the past two decades or threatens to increase in the near future.” (Centers for disease control and prevention 1994). These pathogens include both completely novel infectious agents recently discovered and characterized as human pathogens (for examples see Desselberger 2000) and diseases where human pathogenesis is well

established but incidence is increasing for any number of reasons (for examples see Pfaller & Diekema 2004; Jackson et al. 2000) (Morens et al. 2004). Table 1-1 provides a partial list of emerging human pathogens.

The devastation that infectious diseases have on human health and life expectancy coupled with the threat of novel emerging and re-emerging human pathogens demands scientific investigation into the mechanisms by which diseases emerge. Such an understanding would facilitate the development of procedures and practices that curtail the evolution and spread of human pathogens. Current scientific theory postulates that diseases emerge when pathogens evolve increased virulence towards a host. Within this paradigm, the elucidation of factors that contribute to pathogen evolution involve two types of studies: experimental evolution or serial passage studies within a pathogen-host system and comparative analyses between the genomes of pathogens and closely related nonpathogenic relatives. These studies have revealed three common themes in pathogen evolution: (1) virulence gene acquisition (2) association and host jumping and (3) genome decay. Gaining a more thorough understanding of these factors will allow the prediction and prevention of the evolution of virulent pathogens and the emergence of infectious diseases.

Table 1-1: Examples of emerging human pathogens

Type	Agent	Disease
Prions		
	BSE agent	Creutzfeldt-Jakob disease variant
Viruses		
	Astrovirus	Diarrhea
	Dengue virus	Dengue fever; Dengue hemorrhagic fever
	Ebola virus	Ebola hemorrhagic fever
	Hantaan virus	Hemorrhagic fever with renal syndrome
	Hendravirus	Meningitis, encephalitis
	Hepatitis virus	Hepatitis
	Human herpesvirus	Exanthema subitum, pityriasis rosea, Kaposi's sarcoma
	Human immunodeficiency viruses-1, -2	Acquired immunodeficiency syndrome
	Human T-cell lymphotropic virus-1	Adult T-cell leukemia/lymphoma, tropical spastic paraparesis/HTLV-1 associated myelopathy
	Influenza A virus (H5N1)	Influenza (Hong Kong)
	Lassa virus	Lassa hemorrhagic fever
	Marburg virus	Marburg hemorrhagic fever
	Measles virus	Measles
	Monkeypox virus	Monkeypox
	Nipahvirus	Meningitis, encephalitis
	Poliovirus	Polio
	Puumala virus	Nephropathia epidemica
	Rotaviruses	Infantile diarrhea
	Sabio virus	Brazilian hemorrhagic fever
	Sin nombre virus	Hantavirus pulmonary syndrome
	West Nile virus	Encephalitis (New York)
Bacteria		
	<i>Bordetella pertussis</i>	Whooping cough
	<i>Borrelia burgdorferi</i>	Lyme disease
	<i>Brucella melitensis</i>	Brucellosis
	<i>Camplobacter jejuni</i>	Diarrhea
	<i>Chlamydia trachomatis</i>	Chlamydia
	<i>Corynebacterium diphtheriae</i>	Diphtheria
	<i>Erlishia chaffeensis</i>	Human ehrlichiosis
	<i>Escherichia coli</i> O157:H7	Hemorrhagic colitis, hemolytic uremic syndrome
	<i>Helicobacter pylori</i>	Gastritis, gastric ulcers, increased risk of gastric cancer
	<i>Haemophilus influenzae</i>	Meningitis
	<i>Legionella pneumophila</i>	Legionnaire's disease
	<i>Listeria monocytogenes</i>	Listeriosis
	<i>Neisseria gonorrhoeae</i>	Gonorrhea
	<i>Pseudomonas aeruginosa</i>	Lung, urinary tract, skin, and blood infections
	<i>Rickettsia prowazekii</i>	Epidemic typhus

	<i>Staphylococcus aureus</i> (MRSA)	Pneumonia meningitis, endocarditis, Toxic shock syndrome, septicemia
	<i>Vibrio cholerae</i> O139:H7	Epidemic cholera
Fungi		
	<i>Aspergillus fumigatus</i>	Aspergillosis
	<i>Candida glabrata</i>	Mucosal and systemic yeast infections
	<i>Coccidioides immitis</i>	Coccidioidomycosis
	<i>Cryptococcus neoformans</i>	Cryptococcosis
	<i>Fusarium oxysporum</i>	Onychomycosis
	<i>Malassezia pachydermatis</i>	Fungemia
	<i>Pneumocystis carinii</i>	Pneumocystis pneumonia
	<i>Trichosporon beigelii</i>	Trichosporonosis, white piedra
Helminths		
	<i>Anisakis simplex</i>	Anisakiasis
	<i>Echinococcus granulosus</i>	Hydatid disease
	<i>Metorchis conjunctus</i>	Metorchiasis
	<i>Schistosoma mansoni</i>	Schistosomiasis
	<i>Trichinella spiralis</i>	Trichinosis
Protozoa		
	<i>Cryptosporidium parvum</i>	Acute enterocolitis
	<i>Encephalitozoon hellem</i>	Keratoconjunctivitis, respiratory infections
	<i>Leishmania donovani</i>	Leishmaniasis
	<i>Plasmodium falciparum</i>	Malaria
	<i>Trypanosoma cruzi</i>	Chagas disease

(Taylor et al. 2001)

General theory of the emergence of novel virulent pathogens

Microbes are classified as nonpathogenic saprobes or as infectious pathogens based on their ability to infect and damage a host. Whereas saprobes gain nutrients by degrading dead organic matter, pathogens possess the capacity to invade and colonize a living host. Pathogens are distinguished from symbionts in that unlike symbiosis, the outcome of a pathogenic microbe-host interaction is damage to the host. Host damage varies greatly in both scope and magnitude depending on the type of microbe and the host, but it is always an outcome of the interplay between microbial action and host immune response (Casadevall & Pirofski 1999; Casadevall & Pirofski 2000). Pathogens may be broadly classified as opportunistic, facultative, or obligate based on host range and virulence. Opportunistic pathogens infect a broad host range, but exhibit low virulence, typically infecting only immunocompromised or damaged hosts. Facultative and obligate pathogens are more virulent and are capable of infecting healthy hosts but possess narrower host ranges. Whereas opportunistic and facultative pathogens can survive as saprobes, obligate pathogens must colonize a living host in order to survive (Scheffer 1991). It is generally theorized that the process of natural selection favors the adaptation of a pathogen to the host environment, including the evolution of increased colonization, immune-system avoidance, virulence, and transmission (Brown et al. 2006; Ebert & Herre 1996; Read 1994). In other words, facultative and obligate pathogens evolve from opportunistic pathogens. Opportunistic pathogens in turn evolve from saprobes when opportunity permits a saprobe to colonize a living host utilizing the same mechanisms that allowed it to efficiently degrade and colonize dead organic matter (Arnold et al. 2007; Scheffer 1991).

Broadly speaking, the emergence of infectious diseases encompasses two steps: the introduction of a pathogen into a novel host and the establishment of the pathogen within that host (Morse 1995). The first step is accomplished through one or more ecological factors that bring a potentially pathogenic microbe into contact with a susceptible host such as changes in climate and weather, ecosystems, human demographics and behavior, international travel and

commerce, technology and industry, inadequate public health measures, poverty, war and famine (Berns & Rager 2000; Morens et al. 2004). Such environmental alterations allow saprobes the potential opportunity to colonize living hosts and established pathogens the necessary access to novel hosts. Second, the microbe must initiate an infection cycle including penetration, colonization, and transmission in order to establish itself within the novel host. Changes in host susceptibility to infection, usually due to immunocompromization but occasionally due to a hyper-sensitive immune response (Casadevall & Pirofski 1999), and/or microbial adaptation contribute to the establishment of an emerging pathogen within a host (Morens et al. 2004).

Although many factors contribute to the emergence of infectious diseases, pathogen evolution is the result of natural selection of genetic variants that exhibit adaptations to a novel host environment. Once a pathogen infects and colonizes a susceptible host, the process of natural selection theoretically favors the most-fit variants in the pathogen population. The most-fit variants are those that are the fastest growing and also the most fecund, and pathogens exhibiting both of these traits will invariably inflict greater damage on the host thus increasing virulence (Brown et al. 2006; Read 1994). However, pathogenesis requires transmission to new hosts, thus natural selection promotes a level of virulence that allows for the greatest transmission. If virulence is too high the host may be killed before the virulent variant can sufficiently replicate for transmission, but if virulence is too low, the pathogen may not generate enough transmissible propagules to initiate another infection cycle (Ebert & Herre 1996). The mode of transmission also impacts virulence, and thus pathogen fitness, since horizontal transmission, vector-borne transmission, and increased environmental (non-host) survival time of the infective propagule all allow for the selection of higher virulence levels (Brown et al. 2006; Walther & Ewald 2004). These theories indicate that virulent facultative and obligate pathogens evolve from less virulent opportunistic pathogens (Scheffer 1991). Obligate pathogens evolve when the cost of developing an obligate host specialization is selected over the cost of retaining a free-living lifestyle (Spanu 2006).

Natural selection leading to pathogen evolution requires genetic variants upon which selection can act. Microbial pathogens have a number of mechanisms by which they generate genetic variation (Figure 1-1). First, microbes frequently accept genetic material from other microbes in a process known as horizontal gene transfer. In this manner, pathogens may acquire genes known as virulence factors that increase their virulence towards a host (Groisman & Casadesus 2005; Ochman & Moran 2001; Wren 2003). Second, genetic variation may arise through de novo mutations that may alter both gene products and gene regulation. During a lengthy association with a host, selection will favor the accumulation of certain mutations, thus increasing pathogen fitness and consequently virulence (Groisman & Casadesus 2005). The evolution of a pathogen during a lengthy association with one host may facilitate the pathogenesis of another closely-related host in a process known as “host-jumping” (Cleaveland et al. 2001). Third, de novo mutations and deletions may occur that eliminate functional genes in a process known as genome decay. Gene loss may be selectively neutral, known as reductive evolution, or adaptive (Groisman & Casadesus 2005; Ochman & Moran 2001; Wren 2003). Furthermore, there is evidence that the stress created by the host immune systems increases the frequency of gene loss and gene rearrangement in the pathogen, while environmental stress may accelerate the rate of horizontal gene transfer (Arnold et al. 2007). These processes are the genetic mechanisms by which microbes generate variants upon which selection can act, leading to pathogen evolution and the emergence of novel infectious diseases.

Genetic mechanisms of pathogen evolution

Acquisition of virulence factors

Studies involving genetic comparison of pathogenic species with closely-related nonpathogenic relatives reveal that the acquisition of virulence factors through horizontal gene transfer is the fastest, most effective mechanism by which virulent pathogenic species evolve. In bacteria, virulence genes may be encoded on genetic elements such as plasmids, transposons, bacteriophages or chromosomal pathogenicity islands that are amenable to lateral transfer

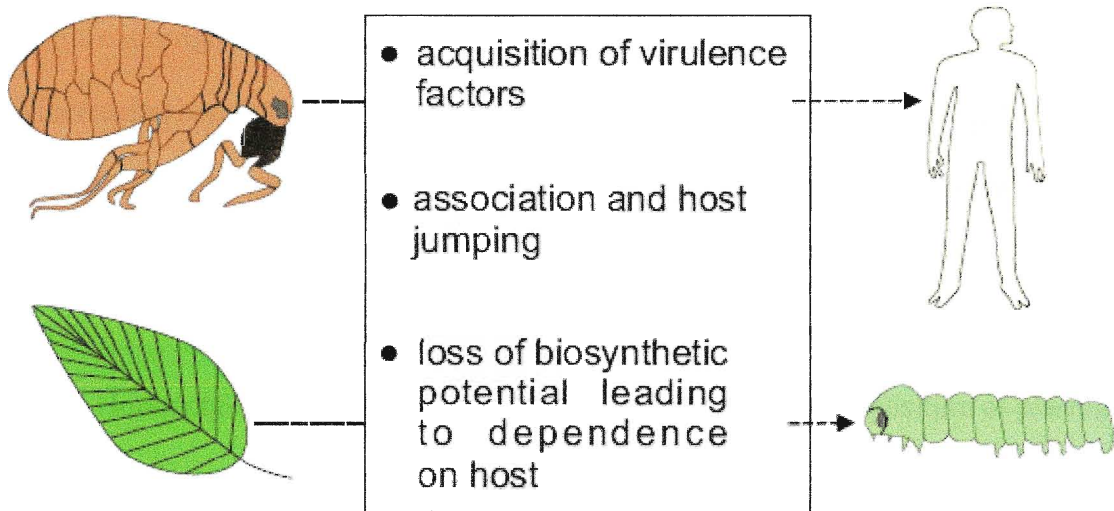


Figure 1-1: Potential mechanisms by which pathogens adapt to alternative hosts, resulting in the emergence of novel diseases. Two examples are shown. The acquisition of virulence-factor-containing plasmids by *Y. pestis* and *B. anthracis* allowed for human pathogenesis. Presumably, both were also able to host-jump to humans due to their ability to evade the innate mammalian immune system potentially as a result of previous association with insects. In addition, their evolution included genome decay, which contributed to their development as obligate pathogens. In the second example, a plant pathogen *A. flavus* probably adapted to insect pathogenesis as a result of close association during insect herbivory. Furthermore, genome decay in the form of a loss of biosynthetic potential and auxotrophy restricted the plant pathogen *A. flavus* to an insect host.

(Hacker et al. 1997), while in viruses, recombination and reassortment are the mechanisms of genetic exchange. Although poorly characterized, horizontal gene transfer occurs in fungi, and an increasing body of evidence implicates such events in fungal evolution. Horizontal gene transfer leading to virulence gene acquisition is a well-documented component of the evolutionary past of many current pathogens. Some of the most striking examples are listed below.

Horizontal gene transfer involving conjugation-mediated acquisition of plasmids containing virulence factors was a crucial step in the evolution of *Bacillus thuringiensis* and *Bacillus anthracis*. *B. anthracis*, the causative agent of anthrax, is one of the species of the *Bacillus cereus* group, a cluster of highly related bacilli that also contains *B. thuringiensis*, a specialized insect pathogen, and *Bacillus cereus*, a mild gastrointestinal human pathogen (Helgason et al. 2000). The vast differences in the pathogenicity and virulence properties exhibited by these species can be attributed to plasmid possession. *B. anthracis* contains two plasmids, pXO1 and pXO2, that carry genes responsible for the production of the anthrax toxin as well as for antiphagocytic activity (Welkos, 1991; Welkos et al. 1993). On the other hand, *B. thuringiensis* contains plasmids encoding an insecticidal protoxin termed δ -endotoxin (Gonzalez et al. 1981). *B. anthracis* and *B. thuringiensis* probably evolved from a *B. cereus*-like ancestor via plasmid acquisition (Waterfield et al. 2004).

Similarly, phylogenetic analysis indicates that *Yersinia pestis*, the causative agent of bubonic plague, emerged from the closely related mammalian enteric pathogen *Yersinia pseudotuberculosis* with which it shares greater than 90% genomic sequence identity (Achtman et al. 1999; Hinnebusch, 1997). A crucial step in *Y. pestis* evolution was the acquisition of two plasmids pPla, containing the plasminogen activator Pla that aids pathogen propagation in the mammalian host, and pFra, encoding the phospholipase D gene Ymt necessary for survival in the flea midgut for future transmission to rats or humans. Interestingly, *Y. pseudotuberculosis* lacks both of these plasmids (Achtman et al. 1999; Hinnebusch et al. 2002; Sodeinde et al. 1992).

Based on genomic comparisons within the *Bacillus* and *Yersinia* genera, virulence factor containing plasmid acquisition is potentially a crucial step in pathogen evolution.

In addition to plasmid acquisition, the transduction of virulence factors via lysogenic phages have also influenced the evolution of human pathogens as evidenced by the emergence of *Escherichia coli* O157:H7 and *Vibrio cholerae* from nonpathogenic progenitors. *E. coli* O157:H7 infection causes severe diarrhea, and in some cases hemorrhagic colitis and hemolytic-uremic syndrome (Brussow et al. 2004). Multilocus enzyme electrophoresis indicates that *E. coli* O157:H7 is closely related to strain O55:H7 known to cause diarrheal disease in infants in part due to the chromosomal *eae* gene product that enables attaching and effacing adherence. Unlike O55:H7, *E. coli* O157:H7 possesses Shiga-like toxin genes encoded by a lysogenic prophage and plasmid-encoded adhesins (Whittam et al. 1993). It appears that *E. coli* O157:H7 emerged from an O55:H7-like ancestor previously adapted for causing diarrhea when transduction with a bacterial virus imparted the *stx* gene for Shiga toxin previously obtained from *Shigella* and conjugation provided plasmid-encoded adherence factors (Wachsmuth et al. 1997; Whittam et al. 1993). Similarly, genetic comparison of human pathogenic strains of the cholera agent *V. cholerae* with nonpathogenic environmental strains, from which they are postulated to have emerged, indicates that many of the differentiating virulence determinants, including the essential cholera toxin (CTX), are encoded by temperate bacteriophages (Faruque & Mekalanos 2003; Waldor & Mekalanos 1996). In addition to *E. coli* O157:H7 and *V. cholerae*, lysogenic conversion following transduction is thought to have been an essential step in the evolution of a number of other bacterial pathogens including *Clostridium botulinum*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Corynebacterium diphtheriae* (Brussow et al. 2004).

Although viruses do not undergo horizontal gene transfer per se, viral virulence can be altered through genetic transfer known as recombination and reassortment. Influenza A virus, the causative agent of human influenza, is a good example of the impact that genetic reassortment has had on the evolution of novel virulent strains capable of initiating human pandemics. Influenza

A virus contains two surface antigens, hemagglutinin (H) and neuraminidase (N), which serve to distinguish strains, mediate infection, and determine strain host range. A human H1N1 strain initiated the human influenza outbreak of 1918, also known as Spanish influenza. The subsequent Asian influenza pandemic of 1957 (H2N2) and the Hong Kong influenza of 1968 (H3N2) occurred following genetic reassortment between reservoir avian influenza A viruses and the endemic human H1N1 viruses descendent from the 1918 pandemic. In both cases, the human H1N1 virus gained alternate hemagglutinin and neuraminidase genes that initiated pandemics due to the immunological naivety of the human population with respect to the hemagglutinin and neuraminidase surface antigens (Horimoto & Kawaoka 2005).

Horizontal gene transfer and its role in transfer of virulence factors and consequently pathogen evolution are well documented in bacteria and viruses. However, evidence indicates that horizontal gene transfer occurs in fungi as well. For instance, comparative genomics indicated that a 14-gene region was transferred from *Cryptococcus neoformans* var. *grubii* to var. *neoformans* ~ 2 million years ago. The transfer was likely the result of parasexual recombination and was mediated by a retrotransposable element (Kavanaugh et al. 2006). Richards et al. (2006) used phylogenetic evidence to identify numerous examples of horizontal gene transfer from filamentous ascomycetes to oomycetes. The genes transferred are thought to have played a role in the development of the similar osmotrophic, filamentous lifestyle of the two groups. The pea pathogen *Nectria haematococca* possesses a supernumerary chromosome containing a pea pathogenicity (PEP) gene cluster including *PDA1*, which confers resistance to the pea antibiotic pisatin. This region displays characteristics similar to bacterial pathogenicity islands such as high similarity to fungal transposases and abnormal codon usage and unusual GC content compared to the rest of the genome, indicating that it is possibly amenable to horizontal transfer (Han et al. 2001). Finally, Friesen et al. (2006) provided evidence of the horizontal transfer of a virulence factor between fungi that significantly enhanced the virulence of a fungal pathogen. Shortly before 1941, the *ToxA* gene of *Stagonospora nodorum*, the causative agent of blotch of

wheat, was transferred to *Pyrenophora tritici-repentis*, resulting in the emergence of tan spot syndrome in wheat. These studies indicate that horizontal gene transfer occurs in fungi and may be a pivotal component of the evolution of fungal pathogens (Rosewich & Kistler 2000).

These examples indicate that for a variety of microbial pathogens, the acquisition of virulence factors through horizontal gene transfer is an important genetic mechanism by which relatively benign microbes can evolve into virulent infectious agents.

Association and host-jumping

Because horizontal gene transfer is capable of affecting a rapid and dramatic impact on pathogen virulence, its role in pathogen evolution compared to de novo mutation events is often over-emphasized. Nevertheless, de novo mutation events that alter or enhance a gene product or gene regulation undoubtedly play a crucial role in pathogen evolution. In fact, horizontal gene transfer appears to be most effective in propelling pathogen evolution when acquired within an accommodating and complementary genetic background. For example, methicillin-resistant *Staphylococcus aureus* evolved from only two of many genetic backgrounds, indicating that its effectiveness as a pathogen is due to the acquisition of antibiotic resistance within a unique genetic background (Oliveria et al. 2002). Theoretically, microbial pathogens constantly generate de novo mutations that are selected for or against during repeated host association, leading to the evolution of virulent, host-adapted pathogens. Although mutations may result in qualitative genetic differences, they may also result in a quantitative fine-tuning of a gene product as has been suggested for the adaptation and speciation of the *Bacillus* genus (Feldgarden et al. 2003). This process of mutation, selection due to host association, and adaptation may also facilitate pathogen evolution through “host-jumping,” whereby a pathogen is successfully transmitted from one host to a completely novel host (Woolhouse et al. 2005).

A variety of serial passage experiments involving the perpetual horizontal propagation of a pathogen through a host population reveal that constant association with a host leads to pathogen adaptation most often noted as increased virulence (Cunfer 1984; Ebert 1998 and

references therein; Hartmann & Wasti 1974; Hayden et al. 1992; Ignoffo et al. 1982; Jinks & Grindle 1963; Mackinnon & Read 1999b; Wasti & Hartmann 1975). The adaptation and increase in virulence is associated with mutations and recombination events (Ebert 1998; Slev & Potts 2002) that have been identified in some cases (Bendinelli et al. 2001; Brown et al. 2001; Chambers & Nickells 2001; Hajimorad et al. 2003; McCullers et al. 2005 Monath et al. 2002). Other studies highlight the aspects of host association that contribute to the selection of virulence-enhancing mutations. For instance, adaptation leading to increased virulence is associated with horizontal (not vertical) transmission (Bull et al. 1991; Stewart et al. 2005) and is usually but not always the result of increased parasitemia and increased transmission rates on the host (Mackinnon & Read 1999a; Ebert 1994). Furthermore, within-host competition and the host immune system are major forces driving pathogen adaptation and the evolution of virulence (Brannan et al. 1994; Ebert & Mangin 1997; Hajimorad et al. 2003; Mackinnon & Read 2004; Taylor et al. 1998; Zhan et al. 2007). These serial passage studies demonstrate that host association results in pathogen evolution through the selection of de novo mutations, culminating in adaptation and increased virulence toward the host.

Evidence exists that in stressful situations, for example the introduction of a pathogen into a hostile host environment, pathogen evolution may be enhanced since microbes can elevate genetic mutation rates in order to generate variants capable of surviving the stressful situation. Hypermutation may be mediated by either a heritable or transient mutator phenotype (Bayliss & Moxon 2002). Studies show that heritable mutators, typically mismatch repair deficient, represent a small minority of many microbial populations and may serve to generate global beneficial mutations that initially allow the microbe to survive in the stressful environment. However, unless recombination separates the beneficial mutations from the mutator phenotype, many deleterious mutations will also accumulate that will be detrimental in the long-term (McKenzie & Rosenberg 2001). Alternatively, transient global hypermutation is a temporary state in which a stressful situation upregulates an error-prone DNA polymerase, which generates

point mutations while repairing stress-induced, double-stranded breaks in the DNA. Since mismatch repair activity is also limited in these situations, mutations accumulate in the genome at a higher than normal rate. When by chance an adaptive point mutation is generated, the stress is removed and hypermutation ceases (Rosenberg 2001). These mechanisms serve to generate de novo mutations at an elevated rate in order to aid pathogen evolution and adaptation in a stressful host environment.

Although it is intuitive that de novo mutations and the subsequent selection imposed by host association is an essential part of pathogen evolution, only a few studies are available that link specific mutations with adaptation. Among them, examples using viral systems are most clearly defined. For instance, during passage of a hybrid yellow fever virus/Japanese encephalitis virus through fetal rhesus lung, a methionine to lysine amino acid mutation in the envelope protein occurred that enhanced neurovirulence toward mice and monkeys but reduced monkey viscerotropism (Monath et al. 2002). Passage of a culture-adapted strain of feline immunodeficiency virus through cats resulted in broad neutralization resistance due to a single amino acid change in the surface glycoprotein (Bendinelli et al. 2001). Similarly, a single amino acid alteration in the matrix protein M1 of influenza B virus during serial passage through mice resulted in increased virulence toward mice (McCullers et al. 2005). In *E. coli* bacteria, random point mutations in the fimbriae gene *fimH* alter its binding properties, causing a shift from a commensal to an urovirulent phenotype (Sokurenko et al. 1998). Intragenetic recombination altering the number of 102bp-tandem repeats in the avirulence gene *pthA* of *Xanthomonas citri* altered its host range with respect to a variety of plants (Yang & Gabriel 1995). In fungi, certain genetic rearrangements among the adhesion gene family members of *Candida* spp. increased their infectivity and virulence toward humans (Verstrepen et al. 2004). Finally, the emergence of a variety of plant pathogenic fungi has been attributed to the gain of toxin genes, presumably derived via mutations, recombination, and selection acting on secondary metabolite biosynthesis genes (Scheffer 1991). These studies show that specific genetic alterations deriving from de

novo mutations or recombination can alter pathogen virulence, tissue tropism, or host range indicative of pathogen evolution.

The generation and selection of genetic changes leading to pathogen adaptation during constant association with a particular host may also play a role in the emergence of novel diseases through host-jumping by conferring upon the pathogen the genetic elements required for a “jump” to another host. For example, the recently emerged human respiratory pathogen *Legionella pneumophila* demonstrated a well-established parasitic relationship with its environmental protozoan reservoir. The properties that enabled *L. pneumophila* to infect and survive in human macrophages are postulated to be a pre-adaptation following an association with a protozoan. Thus, the adaptation that permitted *L. pneumophila* to infect protozoa facilitated its jump to mammalian cells (Harb et al. 2000). Beyond the adaptations that allow a host-jumping pathogen to cause infection in a novel host, host jumping also requires ecological changes (see above) that bring the potential pathogen into contact with the novel host. In order for such opportunistic infections to progress to a serious endemic disease, the pathogen must be sufficiently virulent and transmissible within the new host population, which requires further adaptation through any number of mechanisms (Woolhouse et al. 2005).

Studies show that host jumping to humans following a lengthy association with an animal host is likely an important component of the emergence of novel human diseases. A survey of human pathogens indicated that most current and emerging human pathogens are zoonotic. The fact that 868 of the 1415 (61%) of human pathogens and 132 of the 175 (75%) emerging human pathogens are zoonotic (Taylor et al. 2001) indicates that multihost infection and host-jumping may be an important mechanism by which diseases emerge. Zoonotic pathogens are almost twice as likely to be emerging human pathogens as nonzoonotic pathogens (Taylor et al. 2001), and pathogens that infect more than one species, or more than one order of animal, are more likely to evolve into an emerging disease-causing agent (Cleaveland et al. 2001). Major diseases that have plagued humanity throughout recent history such as smallpox, influenza, tuberculosis,

malaria, measles, and pertussis probably evolved following a lengthy association with animals as pathogens (Pearce-Duvert 2006; Wolfe et al. 2007). Furthermore, genomic studies indicate that the closest free-living relatives of human pathogens *Y. pestis*, *B. anthracis*, and *Photothabdus asymbiotica* express genes that fragment insect tissues and evade the insect innate immune system, indicating that the evolutionary history of these pathogens may have involved ancestors that maintained parasitic associations with insects. Association and subsequent adaptation of these ancestors as insect pathogens may have aided the evolution of the current human pathogens due to a previously acquired ability to degrade animal tissue and evade the innate immune system, processes which are similar in insects and humans (see below) (Waterfield et al. 2004). Thus, human pathogens may emerge through host-jumping due to the genetic adaptations that they acquire during association with another animal host that confer upon the pathogen the ability to infect and colonize humans.

Therefore, in addition to the acquisition of virulence factors through horizontal gene acquisition, pathogen evolution also involves the generation of de novo mutations that are subject to natural selection during association with a host. This process contributes not only to increased virulence of the pathogen toward the associated host but may also enable the pathogen to successfully infect and colonize novel hosts in which adaptations acquired during association with the first host confer an advantage.

Genome decay

In addition to the acquisition of genes by horizontal transfer and de novo mutations that modify microbial DNA, pathogen evolution also involves genome decay. Genome decay involves the silencing or deletion of genes. Genome decay may be neutral, termed reductive evolution, or advantageous, known as pathoadaptation (Maruelli 2007).

Reductive evolution is a common characteristic of obligate pathogens. Presumably deleterious mutations accumulate in their biosynthetic genes, however, these mutations are selectively neutral since obligate pathogens reside solely within their host, obtaining the

necessary nutrients and biosynthetic compounds from their hosts (Wren 2000). Indeed, a variety of obligate pathogens exhibit exceedingly small genomes rife with inactive pseudogenes, including *Chlamydia trachomatis* (Stephens et al. 1998), *Treponema pallidum* (Fraser et al. 1998), and *Rickettsia prowazekii* (Andersson & Andersson 2000). Genetic comparison of some obligate pathogens with close relatives indicates that reductive evolution is an important component of obligate pathogen evolution. *Mycobacterium leprae*, the causative agent of leprosy, possesses a plethora of pseudogenes that are active in the closely-related *Mycobacterium tuberculosis* (Brosch et al. 2000; Cole 1998). Similarly, *Y. pestis* exhibits at least 149 pseudogenes whose homologues are functional in *Y. pseudotuberculosis*, from which it evolved (Wren 2003). The fact that reductive evolution is a common theme among obligate pathogens indicates that selective pressure may favor a minimal genome even if the individual mutations are selectively neutral (Wren 2000).

Although many instances of genome decay are undoubtedly selectively neutral, various studies indicate that sometimes genetic inactivation is advantageous, resulting in increased virulence. In these cases, pathoadaptation is mediated by deletions or point mutations that suppress expression of antivirulence genes (Maurelli 2007). Species comparison shows that whereas *E. coli* strains possess lysine decarboxylase activity and the associated gene *cadA*, *Shigella* spp. uniformly lack lysine decarboxylase due to deletion of *cadA*. This deletion is considered an essential step in the evolution and virulence of *Shigella* since transformation with *cadA* attenuates *Shigella flexneri*, and cadaverine, a byproduct of lysine decarboxylase activity, inhibits *Shigella* enterotoxin (Maurelli et al. 1998). In addition, *Shigella* lacks the *ompT* gene, which is present in *E. coli* K-12. Since the OmpT protease degrades VirG, a surface protein required for the deposition of actin necessary for the spread of *Shigella* to adjacent host intestinal epithelial cells, the deletion of *ompT* via the excision of a lambdoid phage structure was probably an essential step in the evolution of the *Shigella* pathogen (Nakata et al. 1993). Pathoadaptive genome decay during the evolution of *Y. pestis* probably included disruption of putative insect

toxin genes that might kill its flea vector (Wren 2003) and inactivation of *inv* and *yadA*, which encode virulence factors in *Y. pseudotuberculosis*. Deletion of *inv* and *yadA* in *Y. pseudotuberculosis* results in plague-like virulence, while transformation with *yadA* attenuates *Y. pestis* (Rosqvist et al. 1988). The divergence of *Burkholderia pseudomallei*, the causative agent of melioidosis, from the nonpathogenic *Burkholderia thailandensis* likely involved the loss of arabinose assimilation capacity since *B. pseudomallei* lacks the arabinose operon and introduction of the functional *B. thailandensis* arabinose operon reduced virulence (Moore et al. 2004). Beyond comparative genomics, experimental manipulation also shows that gene deletion can increase pathogen virulence since the deletion of two-component regulatory systems in *M. tuberculosis* increased virulence toward mice (Parish et al. 2003). Similarly, adaptation of the malaria parasite *Plasmodium falciparum* to squirrel monkeys during serial passage involved deletion of the ring-infected erythrocyte surface antigen gene *RESA* (Hinterberg et al. 1995), and phage T7 grown on *E. coli* gained replication advantages by deleting a variety of genes whose products can be obtained from the host, including T7 RNA polymerase (Yin 1993).

Thus, pathogen evolution involves genome decay, which may be reductive evolution, eliminating genes for products supplied by the host, or pathoadaptive, yielding an increase in virulence. While reductive evolution is common among obligate pathogens, pathoadaptation is found among both obligate and facultative pathogens.

Future directions: *A. flavus* infectious in *G. mellonella* larvae as a model pathogen-host system for the study of pathogen evolution

Among the many factors that contribute to the emergence of novel diseases in humans, the genetic changes that cause pathogen adaptation and evolution are of critical importance. A variety of studies concur that these genetic changes include virulence gene acquisition through horizontal transfer, de novo mutations, and genome decay. The natural selection of pathogen variants exhibiting beneficial genetic alterations occurs during host infection, leading to the evolution of virulent pathogens. The majority of studies from which these tenets of pathogen

evolution are derived involve examination, genetic manipulation, and occasionally serial passage of current facultative or obligate pathogens based on genetic comparison with nonpathogenic relatives. However, studies attempting to experimentally replicate the evolutionary steps required to generate a novel, virulent pathogen are nonexistent. Such studies would not only confirm or deny the essential elements of pathogen evolution proposed by investigations of known pathogens, but they might also highlight other significant aspects.

A. flavus infections in *G. mellonella* larvae represent an ideal pathogen-host model system for the study of pathogen evolution and the emergence of novel diseases. *A. flavus* is an opportunistic pathogen, in contrast to the facultative and obligate pathogens that are almost exclusively examined with respect to emerging diseases. As an opportunistic pathogen, it has the theoretical potential to evolve increased virulence and/or host dependence indicative of facultative and obligate pathogenesis given the appropriate manipulation. *G. mellonella* larvae are ideal hosts not only because they are easily manipulated, but they are also capable of providing similar selection pressures to pathogens as other animal (human) hosts due to fundamental similarities in their defensive immune responses. Furthermore, such a system is amenable to genetic transformation simulating horizontal virulence gene transfer, host association through serial passage, and genetic dissection to identify instances of de novo mutation and genome decay. For these reasons (outlined extensively below), studies involving *A. flavus* infections in *G. mellonella* will contribute to the understanding of pathogen evolution and disease emergence.

THE OPPORTUNISTIC FUNGUS *A. FLAVUS* AS AN ENTOMOPATHOGEN FOR THE STUDY OF EMERGING DISEASES

The experimental study of emerging diseases requires the selection of an appropriate pathogen-host system that is scientifically well-characterized, subject to laboratory manipulation and analysis, and shares commonalities with other pathogen-host systems such that the findings can be reasonably extrapolated to create implications for the emergence of pathogens toward other hosts. Entomopathogenic fungi and their insect hosts represent such a system. Insect-pathogenic fungi such as *Metarhizium anisopliae* have been extensively studied for the purpose of biocontrol, the practice of using biological agents to inhibit or kill insect pests of agricultural crops. Consequently, the general characteristics, the pathogenic cycle, and the virulence factors of these fungi are well known. Furthermore, entomopathogenic fungi are readily cultured and genetically modified, and susceptible insect hosts are easily inoculated and monitored in a laboratory setting. Also, fungal entomopathogens exhibit the same basic elements of pathogenesis common to all pathogens, namely adherence to the host, invasion of the host tissues, colonization and establishment within the host including evasion of the immune system, infliction of damage to the host often via toxin production, and transmission to new susceptible hosts.

A. flavus was chosen as the ideal pathogen for the study of emerging diseases. *A. flavus* shares many of the same characteristics and pathogenic properties with the well-characterized entomopathogens such as *M. anisopliae*. However, *A. flavus* is an opportunistic pathogen, exhibiting a characteristic broad host range but low virulence, making it similar to other types of opportunistic pathogens from which virulent facultative and obligate pathogens are postulated to emerge (Scheffer 1991).

Entomopathogens

A large number of different fungi cause fungal diseases in insects, and insects from virtually all insect orders are susceptible to a fungal disease. Of the over 700 species of known

insect-pathogenic fungi (Hajek & St. Leger 1994), *M. anisopliae* is well-understood at the genetic and molecular level because it is frequently employed as a biocontrol agent (Roberts & St. Leger 2004). Although commonly found in soil where it grows saprobially and replicates asexually through the production of conidia (Figure 1-2), it is a facultative insect pathogen, showing a strong propensity and proficiency at infecting and killing a variety of different species of healthy insects. In a laboratory setting, *M. anisopliae* is readily cultured on agar media from which its conidia may be harvested and used to infect insects for the general, genetic, and molecular characterization of the entomopathogenic cycle. Since most entomopathogens appear to behave the same as *M. anisopliae* during insect infection, it is from this species of fungi that general knowledge of the pathogenic cycle of entomopathogens is derived.

Pathogenic cycle

As with all pathogens, entomopathogens progress through the basic infection stages of adhesion to the host, invasion of the host, dissemination within the host including immune system avoidance, destruction of the host often through toxin production, and transmission to new hosts (Figure 1-3).

Adhesion

The conidia of entomopathogenic fungi are the infective propagules. During the initial stages of pathogenesis, conidia attach to the insect cuticle via non-specific hydrophobic interactions between the conidial wall and the waxy lipid layer of the epicuticle (Boucias et al. 1988). Rodlet proteins called hydrophobins present in the infection structures of *M. anisopliae* (St. Leger et al. 1992b) are responsible for the hydrophobic nature of the conidia and thus adherence to the insect cuticle (Boucias et al. 1988; Bidochka et al. 2001).

Following adherence, the conidia germinate utilizing the lipids and trace amounts of soluble nutrients present on the cuticular surface. Germinated conidia form short germ tubes that differentiate terminally into appressoria or “hold-fast” structures (Clarkson & Charnley 1996). Appressoria formation is induced by both the hard, hydrophobic nature of the cuticle and the

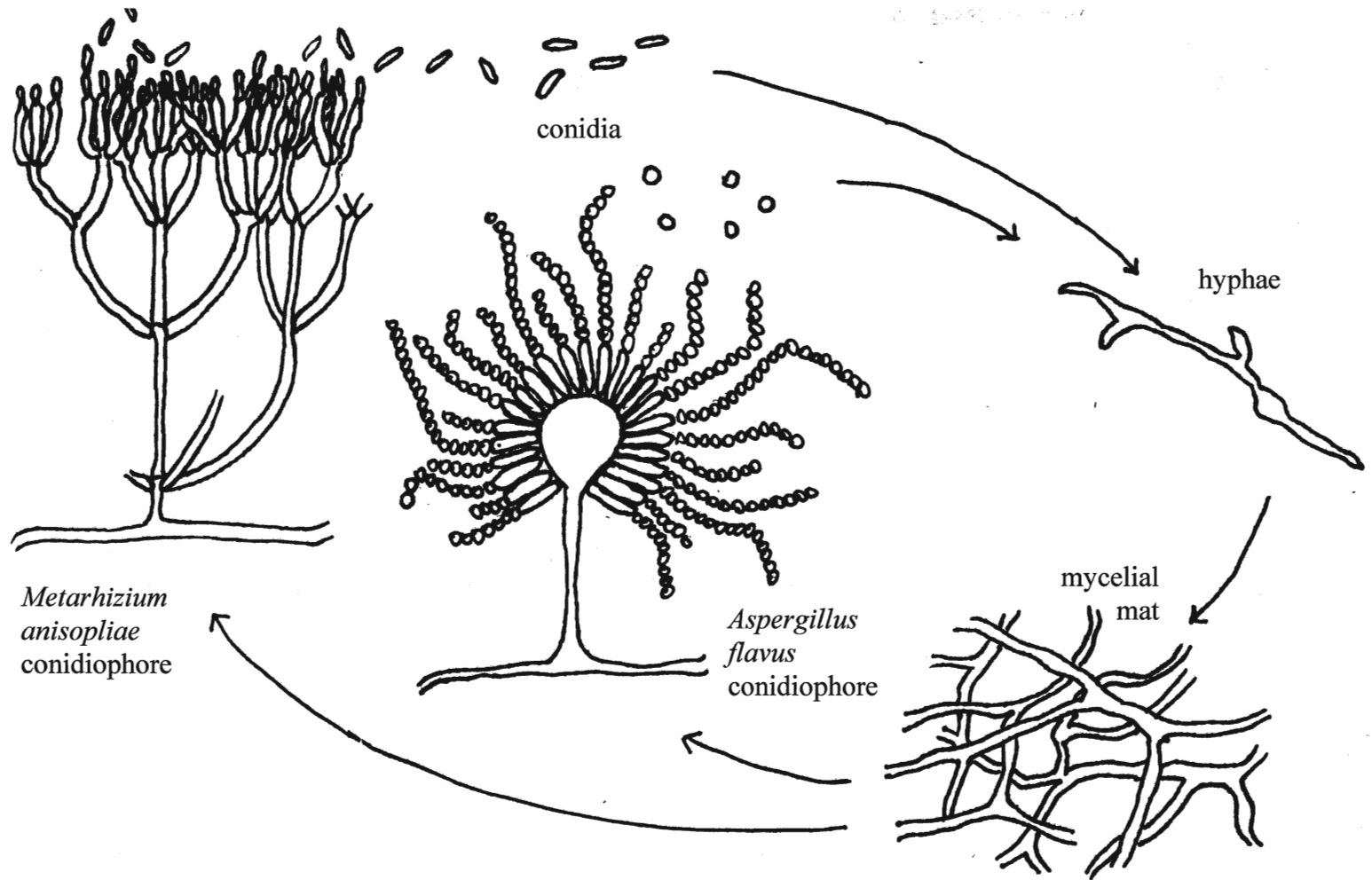


Figure 1-2: Life cycle of the asexually reproducing fungi *M. anisopliae* and *A. flavus*. Conidia germinate forming hyphae, which grow apically and branch extensively to generate a mycelial mat. Conidiophores differentiate from the mycelia and produce conidia, which are wind-dispersed to new locations.

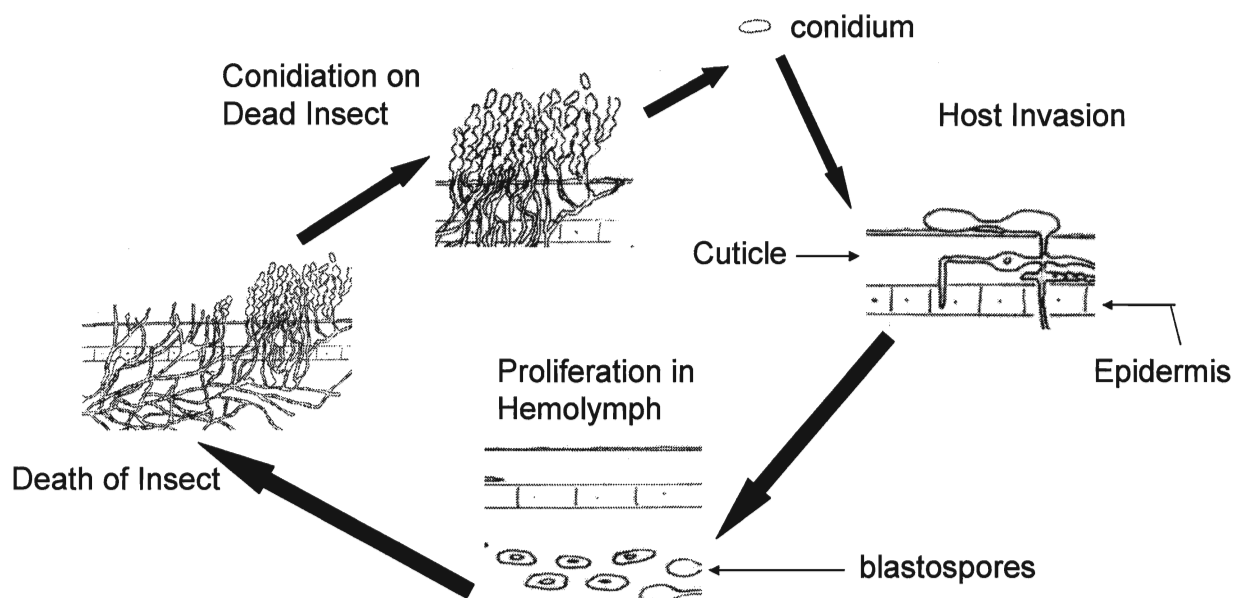


Figure 1-3: Schematic diagram demonstrating the different stages of the infection cycle of entomopathogens. After landing on the insect host cuticle, conidia germinate, producing short germ tubes and terminal appressoria. The appressoria secrete an arsenal of proteolytic enzymes that degrade insect cuticle, allowing access to the hemocoel. Growing as blastospores in the hemolymph, the fungus spreads throughout the insect body, producing toxins and killing the insect. Upon death of the host, the fungus ramifies through the cadaver, eventually emerging to conidiate on the surface. Conidia are wind-dispersed to new hosts. This diagram is courtesy of Cherrie-Lee Small and Jonathon LeBlanc.

low levels of nitrogenous compounds present due to a scant mixture of amino acids on the cuticle (St. Leger et al. 1989a).

Host invasion

The appressoria produces a penetration peg that breaches the cuticle, allowing the fungus to access the nutrient-rich hemocoel. In addition to the mechanical pressure applied by the growing penetration peg, the appressoria secretes an arsenal of proteases and chitinases that enzymatically degrades the protein and chitin components of the cuticle (Clarkson & Charnley 1996). Beyond aiding penetration, these enzymes provide the growing fungus with nutrients (St. Leger et al. 1989b). The enzymes include subtilisin-like (Pr1) and trypsin-like (Pr2) endoproteases (St. Leger et al. 1987a), a metalloendoproteinase (St. Leger et al. 1994b), exopeptidases, including a carboxypeptidase (St. Leger et al. 1994a) and several amino peptidases (St. Leger et al. 1995), esterases, and exochitinases (St. Leger et al. 1987b).

The major protein secreted by the appressoria is the endoprotease Pr1 (St. Leger et al. 1989b), which also incidentally shows considerable activity towards insect cuticle (St. Leger et al. 1987a). Gold-labeled rabbit antisera to Pr1 confirmed Pr1 secretion by the appressoria during *M. anisopliae* penetration of the insect cuticle and that penetration requires both enzymatic degradation as well as mechanical pressure (Goettel et al. 1989). In correlation with its secretion from appressoria, which form under low nutrient conditions (St. Leger et al. 1989b), Pr1 is also secreted during nutrient deprivation (St. Leger et al. 1991b), specifically when lacking a readily utilizable carbon and nitrogen source (St. Leger et al. 1989a).

Pr1 is a chymoelastinolytic, subtilisin-like, serine protease (St. Leger et al. 1987a; St. Leger et al. 1992a). The cDNA of *pr1* has been cloned, indicating that it is synthesized from a 41kDa precursor protein translated from a 1164bp mRNA sequence. The large precursor contains a signal sequence, a propeptide, and the 28.6kDa mature protein. Characteristic of subtilisin-like proteases, the active site contains a catalytic triad of aspartate, histidine, and serine (St. Leger et al. 1992a). The active site accommodates at least five residues with affinity toward

hydrophobic moieties containing alanine, proline, and phenylalanine, which are abundant in the insect cuticle (St. Leger et al. 1987a; Paterson et al. 1994).

Correlated with a high isoelectric point ($pI > 10$) (St. Leger et al. 1987a), Pr1 has a proportionately large number of positively charged, basic amino acids on its surface (St. Leger et al. 1992a). Consequently, Pr1, as a basic protease, electrostatically adsorbs onto insect cuticle by non-specific attraction to the negatively charged carboxyl or hydroxyl groups of the glutamic acid and aspartic acid residues prevalent in insect cuticle (St. Leger et al. 1986; Bidochka & Khachatourians 1994). The adsorption of Pr1 onto the insect cuticle appears to be separate and independent from proteolytic hydrolysis (St. Leger et al. 1986), and it is this high affinity for insect cuticle that makes Pr1 significantly more efficient than other subtilisin-like proteases, such as Proteinase K of *Tritirachium album*, at degrading insect cuticle (St. Leger et al. 1992a).

The important role of Pr1 in the pathogenesis of *M. anisopliae* was first demonstrated by St. Leger et al. (1988a) who showed that treatment of an insect host with specific Pr1 inhibitors decreased the virulence of *M. anisopliae*. Furthermore, spontaneous *pr1*-deficient mutants of *M. anisopliae* exhibited decreased virulence towards certain insect hosts (Wang et al. 2002). Conversely, overproduction of Pr1 in *M. anisopliae* decreased survival time of inoculated *Manduca sexta*, indicating the potential of Pr1 to increase the virulence of entomopathogenic fungi and confirming the importance of Pr1 to insect pathogenesis (St. Leger et al. 1996).

The most recent characterization of the Pr1 cuticle-degrading protease of *M. anisopliae* indicates that multiple isoforms are encoded in the genome and that 11 subtilisins (designated Pr1A-K) are expressed during growth on insect cuticle (Bagga et al. 2004). Besides different amino acid sequences, at least some of these isoforms demonstrated different isoelectric points (St. Leger et al. 1994b). The Pr1 protease cloned and characterized as described above has been designated Pr1A, and it is by far the most abundant isoform secreted by *M. anisopliae* during growth on insect cuticle (Bagga et al. 2004).

Dissemination within the host

Once *M. anisopliae* enters the hemolymph, growth occurs as yeast-like blastospores or short hyphal bodies rather than mycelia. The blastospores effectively disseminate throughout the insect hemocoel, obtaining nutrients from the hemolymph (Clarkson & Charnley 1996).

Immune system avoidance

The basic immune responses mounted by the insect toward the invading fungus are phagocytosis and encapsulation, melanization, and antimicrobial peptide production. Phagocytosis and encapsulation are accomplished by the insect hemocytes as they ingest or contain the growing fungus. The phenoloxidase cascade in the insect hemolymph produces melanin, which is toxic and inhibitory to fungi. A range of antimicrobial peptides released by the insect fat body following infection also target and kill the fungal pathogen (Gillespie et al. 2000; Kimbrell & Beutler 2001). Nevertheless, *M. anisopliae* efficiently evades these immune responses. Proteases of *M. anisopliae* inhibit the spreading, attachment, and phagocytosis of insect plasmatocytes, presumably aiding immune system evasion during infection (Griesch & Vilcinskas 1998). The blastospores or hyphal bodies present in the hemocoel are covered with an antiadhesive, collagenous protein that provides protection from hemocytes by hiding the immunogenic β -1,3-glucans of the fungal cell wall (Wang & St. Leger 2006). Furthermore, *M. anisopliae* produces toxins known as destruxins that suppress antimicrobial peptide production (Pal et al. 2007) and alter hemocyte structural components thereby decreasing encapsulation and phagocytotic efficacy (Vey et al. 2002; Vilcinskas et al. 1997).

Host damage

Destruction of the host by the invading fungus includes both mechanical damage due to fungal growth in the hemocoel and toxin production (Clarkson & Charnley 1996). *M. anisopliae* produces a family of peptide toxins known as destruxins. Destruxins injected into the hemocoel are toxic to lepidopteran larvae, causing tetanic paralysis of a variety of tissues including the midgut, Malpighian tubules, hemocytes, and muscles. Paralysis is apparently due to the

activation of Ca^{2+} ion channels, leading to the membrane depolarization (Samuels et al. 1988).

There is a strong positive correlation between destruxin production and virulence indicative of its role in insect pathogenesis, however, there are highly virulent strains of *M. anisopliae* that do not produce destruxins (Kershaw et al. 1999).

Transmission

Following the death of the insect, the fungus continues to grow on the cadaver eventually emerging from the dead host to conidiate on the external surface. Conidia are wind-dispersed to new susceptible hosts (Shah & Pell 2003).

A. flavus as an entomopathogen

Although the *Aspergillus* species replicate asexually similar to *M. anisopliae* (Figure 1-2), the two taxa differ in that while *M. anisopliae* is a facultative insect pathogen, the *Aspergillus* species are characteristic opportunistic fungal pathogens. A number of species from this genus are reported to cause infections in plants, insect, and mammals, although the vast majority of infections are subsequent to damage or immunocompromization of the host. *A. flavus* is a major contaminant of economically important crops such as corn and peanuts, while *Aspergillus fumigatus*, and at a lesser frequency *A. flavus*, cause invasive aspergillosis in the human lung (Yu et al. 2005). Other species such as *A. nidulans* demonstrate a saprobic lifestyle. Of the opportunistic *Aspergillus* species, *A. flavus* exhibits a particularly broad host range. Unlike *A. fumigatus* and *Aspergillus nidulans*, *A. flavus* is able to infect living plant and insect tissues (St. Leger et al. 2000) in addition to its implication in some cases of invasive aspergillosis in humans (Yu et al. 2005). In conjunction with its broad host range, *A. flavus* exhibits a broader spectrum of protein and polysaccharide degrading enzymes than facultative plant and insect pathogens, including proteases, elastases, mucinases, chitinases, cellulases, pectinases, sodium polypectinases, xylanases, and cutinases (St. Leger et al. 1997). With respect to plant, insect, and mammalian hosts, the opportunist *A. flavus* exhibits a characteristic low virulence and relatively low incidence of infection compared with facultative pathogens of these hosts.

A. flavus is the *Aspergillus* species most often reported to infect insects (Campbell 1994) and has been documented as an insect pathogen from a variety of insects, including silkworms (*Bombyx mori*) (Patil 1988), lace bugs (*Stephanitis typical*) (Sathiamma et al. 1988), rice grasshoppers (*Oxya nitidula*) (Ambethgar 2002), caterpillars (*Opisina arenosells* W.), plant hoppers (*Proutista moesta* Westwook) (Gupta & Gopal 2002), and mosquitos (*Anopheles stephensi*) (Sur et al. 1999). Furthermore, microscopic examination of the infection process revealed extensive similarity to the well-characterized infection process of the facultative insect pathogen *M. anisopliae*. *A. flavus* naturally infects the German cockroach, *Blattella germanica*, causing death 48-72 h after infection. Microscopic examination of the cadavers revealed fungal growth in the hemocoel with granulocytes and plasmatocytes engulfing the invading fungus. Presumably, *A. flavus* gained entry into the host via penetration through the insect cuticle (Kulshrestha & Pathak 1997). An extensive surface ultrastructural study followed the pathogenic cycle of *A. flavus* on *B. mori* documenting the germination of the conidium on the cuticle, the formation of appressoria after 24h, the entrance of hyphae into the hemocoel 2 days post-inoculation, fungal multiplication and the death of the larvae after 4-5 days, ramification of the fungus throughout the hemocoel, and the extensive fungal outgrowth including the formation of aerial conidiopores on the cadaver surface 6-7 days after inoculation (Kumar et al. 2004).

Studies on the insect pathogenicity of *M. anisopliae* revealed that the secretion of cuticle-degrading enzymes, specifically proteases, and the production of toxins are two important sets of virulence factors that aid entomopathogenicity. *A. flavus* secretes both proteases and chitinases, which presumably assist in penetrating the insect (St. Leger et al. 1997). Among these proteases, *A. flavus* produces a protease with an isoelectric point of ~pH 8 that degrades a bulky peptide substrate containing hydrophobic amino acids preferred by Pr1 of *M. anisopliae* (St. Leger et al. 2000; St. Leger et al. 1987a). Specifically, *A. flavus* secretes two proteases: an alkaline serine protease and a metalloprotease. The 35kDa alkaline protease exhibits elastinolytic activity and possesses the catalytic triad of aspartate, histidine, and serine indicative of a member of the

subtilisin family of proteases (Ramesh et al. 1994). Also displaying elastinolytic activity, the 23kDa metalloprotease with an isoelectric point of 9.0 is produced by autolysis of a 35kDa secreted protein and exhibits remarkable pH and temperature stability (Mellon and Cotty 1996; Ramesh et al. 1995; Rhodes et al. 1990). In addition to proteases, *A. flavus* synthesizes metabolites that are toxic to insects. Aflavarin, B-aflatrem, and aflatoxin B1 are all orally toxic to the beetle *Carpophilus hemipterus*, the earworm *Helicoverpa zea*, and the silkworm *B. mori* respectively (Ohtomo et al. 1975; TePaske & Gloer 1992). However, the role of these toxins in insect pathogenicity is ambiguous since both aflatoxin and non-aflatoxin producing strains of *A. flavus* have been isolated from coconut insect pests (Gupta & Gopal 2002).

A. flavus is an ideal model pathogen for the study of emerging diseases within the context of a fungal-insect system. It is a characteristic opportunistic pathogen, secreting a broad range of degradative enzymes that facilitate its equally broad host range. As an insect pathogen it exhibits low virulence often requiring a damaged or immunocompromised host for infection. Thus, it has the potential for adaptation and evolution toward facultative insect pathogenesis. This conjecture is substantiated by the widely held hypothesis that facultative insect pathogens such as *M. anisopliae* evolved from saprobic and opportunistic fungi that employ a variety of enzymes for the degradation dead or living but damaged tissues. This theory links the evolution of insect pathogenesis with nutritional specialization; facultative insect pathogens emerged following the acquisition of specialized enzymes that allowed them to specifically and effectively degrade living as well as dead insect tissue (St. Leger & Bidochka 1996; Shah & Pell 2003).

***A. flavus* as a model pathogen for the study of pathogen evolution**

Fungal pathogens and their associated insect hosts represent an ideal pathogen-host model system for the study of emerging diseases. In addition to easy cultivation and genetic manipulation in a laboratory setting, the characteristics and pathogenic cycle of entomopathogens are well known. Susceptible hosts are readily available and easily infected and monitored. Furthermore, fungal entomopathogens progress through standard phases of infection

and pathogenesis exhibited by all pathogens, making extrapolation to other pathogen-host systems justifiable. Among the entomopathogens, *A. flavus* is an ideal selection for investigating pathogen evolution because it is an opportunistic pathogen, exhibiting low virulence but a broad host range. It demonstrates the potential to evolve increases in virulence and specificity toward insect hosts that can be characterized phenotypically and examined on a genetic and molecular level. Thus, *A. flavus* infections in insects represent a promising and unique approach to the study of emerging diseases.

DEVELOPING INSECT MODELS FOR THE STUDY OF CURRENT AND EMERGING HUMAN PATHOGENS

As indicated above, the study of infectious diseases is a topic of considerable concern regarding human health and well-being. Beyond the vast number of well-characterized human diseases, novel human pathogens appear to be constantly emerging. Consequently, understanding the mechanisms of pathogen evolution and disease emergence is a major focus of research. Although a great deal of knowledge regarding pathogen evolution has been gleaned from the investigation of known human pathogens, this field of study would greatly benefit from the experimental manipulation of a model pathogen-host system in order to recreate essential steps of pathogen evolution. In order to apply the insights gained from such a model system to emerging human pathogens, the chosen host must provide a similar environment with analogous selection pressures as a human host. Currently, most research involving human pathogens utilizes mammalian hosts, including rodent, simian, and feline hosts. Besides the substantial ethical concerns over mammalian suffering, studies using these host systems are time-consuming and incur considerable costs for animal maintenance. Recently a number of researchers have begun to employ insects as model hosts for the study of human-pathogenic microbes instead of mammals (Kavanagh & Reeves 2004; Mylonakis & Aballay 2005; Tan 2002). The promising results of these studies give credibility to the notion that using insects as hosts to study pathogen evolution can provide insights into the genetic and phenotypic mechanisms by which novel human pathogens emerge.

The substitution of insects for mammals as model hosts for the study of pathogen evolution not only has a number of benefits, but it is also scientifically valid. Insects can be employed in large numbers and are easily manipulated, resulting in minimal time and cost in their maintenance. The infection process relative to mammals is much quicker, yielding results more rapidly. Also, there are fewer ethical issues associated with inoculating insects with pathogens than mammals. Moreover, results obtained using insects as model hosts can easily be

confirmed using mammalian models, alleviating concerns over unwarranted extrapolation of the results. Although some incongruities between pathogen evolution in insects and in humans are expected, two arguments validate that the general trends should be the same. First, the commonalities in the infection process and the similarities between human and insect immune responses (Salzet 2001) endorse this model system. Second, insect hosts have been used to accurately assess virulence and identify virulence factors in a number of opportunistic human pathogens, showing good correlation with mammalian host systems (Brennan et al. 2002; Jander et al. 2000). For these reasons, the use of insects such as *G. mellonella* larvae in conjunction with the opportunistic entomopathogenic fungus *A. flavus* as a model host-pathogen system for the study of the mechanisms of pathogen evolution and extrapolation to emerging human diseases is both valid and appropriately beneficial.

Parallels in insect and human pathogenesis

Common themes are repeatedly followed by pathogenic microbes in a disease process toward a host, whether mammalian or insect (Figure 1-4). These themes include the adhesion of the pathogen to the host, entry and invasion of the host, establishment, dissemination within the host, toxin production, avoidance of host immune responses, and transmission. These commonalities reinforce the validity of using insects as model hosts to study the general commonalities of pathogen evolution.

The first line of defense against an invading microorganism is the mammalian epidermis or the insect cuticle. Structurally, the mammalian epidermis and insect cuticle are similar in that the major constituent of each is protein (Figure 1-4a), although there are differences as epidermal tissue lacks the chitin possessed by insect cuticle. During pathogenesis, insect pathogens typically use proteases to degrade intact insect cuticle (St. Leger et al. 1987b), while mammalian pathogens rarely breach the intact epidermis, but utilize arthropod vectors or penetrate damaged skin instead. However, following initial penetration, mammalian pathogens secrete extracellular proteases as virulence factors that aid in tissue destruction, inactivation of host defense

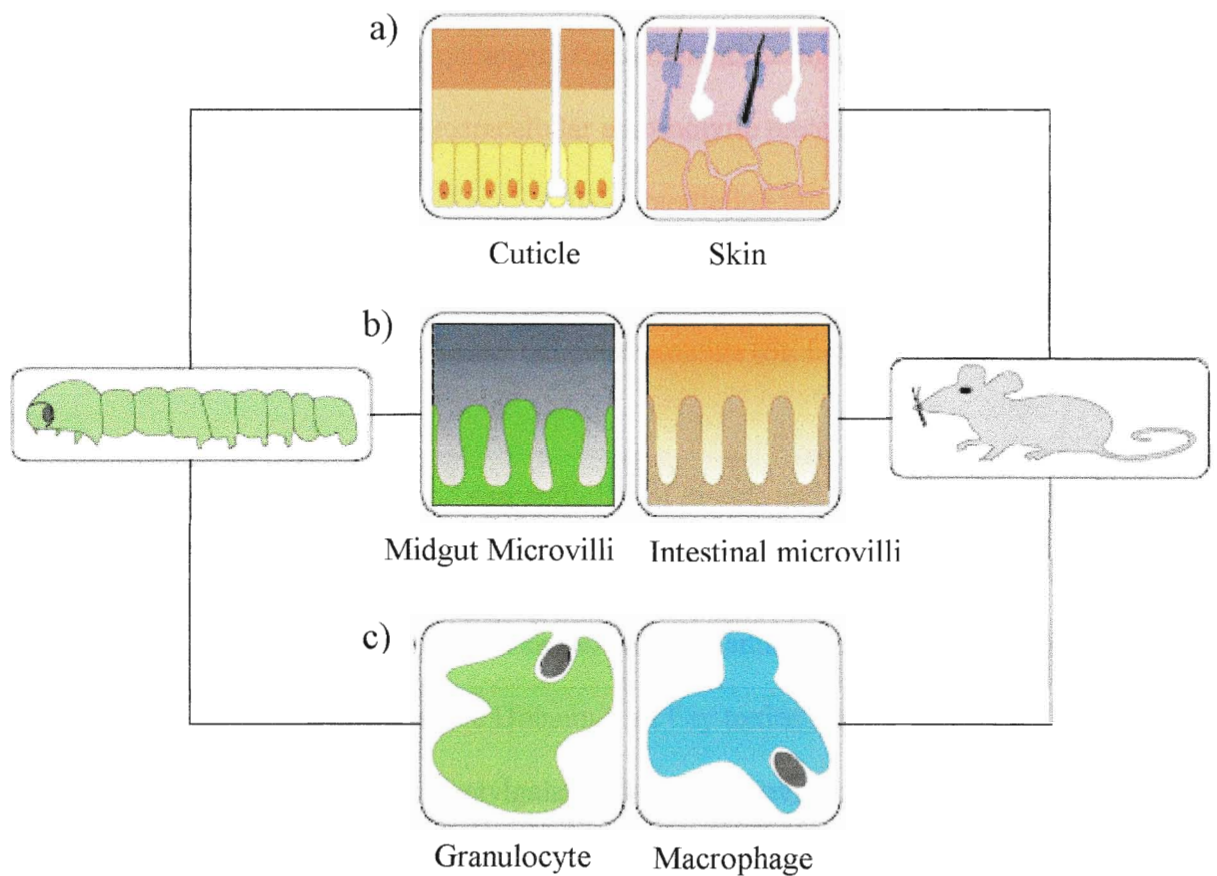


Figure 1-4: Parallels between mammals and insects with respect to microbial infection. (a) During infection, pathogens secrete proteases to degrade the protein component of insect cuticle or compromised mammalian epidermis. (b) Toxins exert their effect by binding to receptors on the intestinal microvilli of mammals or the insect midgut. (c) Foreign invaders are engulfed by granular cells that patrol the insect hemolymph or macrophages circulating in the mammalian blood stream.

molecules, activation of regulatory proteins, and nutrient acquisition (Maeda 1996). For example, the opportunistic human pathogen, *Pseudomonas aeruginosa*, a frequent pathogen of burn victims, secretes at least two extracellular elastinolytic proteases that act as virulence factors at the invasive stage (Cowell et al. 2003). Paralleling this, insect pathogenic fungi such as *Beauveria bassiana* and *M. anisopliae* secrete proteases, in particular a subtilisin-like elastinolytic protease that degrade insect cuticular proteins (St. Leger et al. 1987a; St. Leger et al. 1992a).

Parallels in the detection and susceptibility of insects and mammals towards pathogen toxins are also evident. Glycoconjugate receptors for microbial-derived toxins are found on the microvillar surfaces of the insect midgut as well as the mammalian intestine (Figure 1-4b). During *B. thuringiensis* infections, the Domain II of Cry toxins derived from the parasporal crystal show specificity toward N-acetylgalactosamine microvillar receptors in the insect midgut (Jurat-Guantes et al. 2002). Several other receptors in the insect midgut, including aminopeptidase N (Agrawal et al. 2002) and cadherin-like protein (Hua et al. 2004), have also been implicated as receptors for Cry toxins. Glycoconjugate receptors for bacterial toxins also play a role in mammalian enteric infections. For example, the toxin B portion of cholera toxin CTX recognizes the oligosaccharide moiety of sialic acid found on the G_{m1} gangliosides in the mammalian intestine (Cuatrecasas 1973).

Successful pathogen invasion of a host also requires the evasion of host immune responses. Insects and mammals possess a conserved innate immune system that functions to recognize pathogenic invaders and elicit an appropriate immune response. Lacking adaptive immunity, insects exhibit cellular and humoral innate immune responses. In the cellular response, hemocytes phagocytose, encapsulate, and melanize invading microbes, while antimicrobial peptide secretion from the fat body constitutes the humoral response (Kimbrell & Beutler 2001). Conversely, mammals possess an innate immune response involving phagocytes,

antimicrobial substances, complement proteins, and cytokines as well as an adaptive immune response mediated by the T- and B-lymphocytes resulting in antibody secretion (Beutler 2004). Although differences exist, for example melanization via the prophenyloxidase cascade in insects (Kanost et al. 2004) and the complement system in mammals (Rus et al. 2005), common themes of antimicrobial substance production and phagocytosis (Figure 1-4c) are evident. Indeed, both insect hemocytes and human neutrophils use superoxide to kill phagocytosed microbes and possess homologous proteins for superoxide production (Bergin et al. 2005).

The conserved innate immune system of insects and mammals recognizes foreign invaders in a similar manner and uses analogous signaling pathways to activate major immune responses (Heine & Lien 2003; Hoffmann et al. 1999; Medzhitov 2001; Medzhitov & Janeway 1997). In both hosts, recognition is mediated by distinctive pathogen-associated molecular patterns (PAMPs), which are essential, conserved microbial components such as lipopolysaccharides of gram negative bacteria, peptidoglycan and teichoic acids of gram positive bacteria, mannans of yeast, β -1,3-glucans of fungi, and unmethylated CpG of bacteria (Medzhitov & Janeway 1997). Insect and mammalian innate immune systems possess pattern recognition receptors (PRRs) that recognize PAMPs and mount an appropriate immune response. In some cases, the PRRs of insects and mammals exhibit molecular and functional similarity (Khush & Lemaitre 2000; Kimbrell & Beutler 2001). Furthermore, pathogens of both hosts have evolved mechanisms to circumvent immune system recognition by altering surface components that act as PAMPs. For example, both gram-negative human pathogenic bacteria and the insect fungal pathogen *B. bassiana* modify cell surface components, outer membrane lipopolysaccharide O antigen or cell wall galactomannan composition respectively, to avoid immune system recognition (Pendland et al. 1993; Whitfield and Roberts, 1999).

In *Drosophila*, PAMP recognition by a PRR activates a proteolytic signaling cascade in the hemolymph, which subsequently activates the transmembrane receptor protein Toll. Toll activation then commences an intracellular signaling cascade, resulting in NF κ B-mediated

transcription of antimicrobial peptides (Hoffmann et al. 1999; Medzhitov 2001). Mammals possess a number of transmembrane Toll-like receptors (TLRs) that are homologous to *Drosophila* Toll proteins (Hoffman et al. 1999; Khush & Lemaitre 2000). In mammals, TLRs act as PRRs to mediate adaptive immunity. Located on patrolling dendritic cells, PAMP recognition by TLRs initiates an intracellular signaling cascade analogous to *Drosophila* Toll stimulation (Hoffman et al. 1999), culminating in the secretion of co-stimulatory molecules and cytokines. These activate naïve T cells, which in turn prompt macrophages to kill ingested pathogens and B cells to secrete antibodies (Heine & Lien 2003; Medzhitov & Janeway 1997). Therefore, the signaling pathways following PRR recognition of PAMPs are conserved components of innate immunity that serve to elicit an appropriate immune response whether selective antimicrobial peptide production in insects or a proper adaptive immune response in mammals (Medzhitov & Janeway 1997). Thus, the mechanisms required by pathogens for immune system evasion are similar in insect and mammalian hosts because of the conserved innate immune system that functions to recognize the invading pathogen and mount an appropriate immune response, therefore reinforcing the validity of using insects as models for the study of pathogen evolution for the application to emerging human diseases.

Insects as Models for the Study of Opportunistic Human Pathogens

Beyond the noted similarities between insect and mammalian pathogenesis, insects represent good model hosts for the study of emerging diseases applicable to human pathogen evolution due to the similarities in host response between mammals and insects when infected with opportunistic pathogens. The fact that insects and mammals exhibit similar responses when infected with opportunistic pathogens validates the assumption that insect and mammalian hosts provide similar environments with comparable selection pressures for pathogens. This notion has been substantiated using bioassays of the human opportunistic pathogens *Candida albicans* and *P. aeruginosa* against both types of hosts, demonstrating astonishing similarities in their responses namely a significant positive correlation in virulence.

In bioassays against mice as well as larvae of the wax moth, *G. mellonella* (Lepidoptera), *C. albicans* mutant strains deficient in the ability to transition between yeast and hyphal states demonstrated attenuated virulence with good correlation in relative virulence of the mutant strains between each of the two hosts. It suggests that genes controlling the switch from yeast to hyphal states are important in the pathogenesis of both hosts, indicating that studies with *G. mellonella* are capable of identifying virulence factors required for mammalian pathogenesis (Brennan et al. 2002). Bioassays also showed that *G. mellonella* larvae were susceptible to infection by *C. albicans* as well as other *Candida* species but not to *Saccharomyces cerevisiae* (Cotter et al. 2000). The relative virulence of *Candida* species to *G. mellonella* larvae also correlated well with the relative frequency of human infections by various *Candida* species (Fridkin & Jarvis 1996). For example, *C. albicans* exhibited greater pathogenicity than *C. glabrata* toward *G. mellonella* (Cotter et al. 2000) and also showed a higher frequency of infections in humans than did *C. glabrata* (Fridkin & Jarvis 1996).

Bioassays with *P. aeruginosa* mutant strains showed a significant positive correlation between the percent survival of mice and the LD₅₀ value (50% lethal dose) of *G. mellonella* larvae (Jander et al. 2000). In addition, bioassays with *Drosophila melanogaster* were able to identify the elevated virulence of the Liverpool epidemic strain (LES) of *P. aeruginosa* compared to other strains. The LES is an aggressive, hypervirulent strain implicated in a substantial percentage of *P. aeruginosa* infections of cystic fibrosis patients (Salunkhe et al. 2005). These studies set the precedent for the continued use of insects such as *G. mellonella* larvae and *D. melanogaster* to screen isolates of opportunistic pathogens such as *Candida* and *Pseudomonas* species for relative virulence towards humans. These correlations are only applicable to opportunistic pathogens that readily infect both insects and mammals (Tan 2002) since specialized human pathogens, such as human immunodeficiency virus (HIV) or *M. tuberculosis*, are nonpathogenic towards insects.

Furthermore, putative virulence factors from gene-knockout strains of opportunistic pathogens can be screened in insects before testing in mammals. The relative virulence of gene-knockout strains of *P. aeruginosa* and *Pseudomonas fluorescens* towards *G. mellonella* or *D. melanogaster* have identified several mammalian virulence factors such as *hfq*, an RNA modifier and general virulence regulator (Sonnleitner et al. 2003), *pscD*, ExoT, and ExoU, components of the type III secretion system (Miyata et al. 2003), a *Yersinia ybtQ* homolog presumably involved in iron acquisition (Choi et al. 2002), the *pilGHIJKL chpABCDE* gene cluster involved in twitching motility and signal transduction (D'Argenio et al. 2001), *qscR*, a regulator of quorum-sensing-controlled gene expression (Chugani et al. 2001), and two adherence factors (de Lima Pimenta et al. 2003). In most of the studies, murine hosts were used to confirm the importance of the identified virulence factor for mammalian infection.

Both insect and mammalian hosts demonstrate parallel responses when infected with opportunistic pathogens, indicating that they provide analogous selection pressures and host environments for invading pathogens. These studies indicate that insect host-pathogen systems represent powerful tools for the study of pathogen evolution with general trends applicable to the understanding of the emergence of novel human diseases.

The insect *G. mellonella* larvae as a model host for the study of pathogen evolution

Although it is recognized that novel emerging human diseases could seriously devastate the human population, research regarding pathogen evolution is limited primarily to speculations on the evolutionary past of known human pathogens. A pathogen-host model system that is subject to scientific experimentation could contribute substantially to the understanding of pathogen evolution. Such a system requires a host that is both easily manipulated and exhibits a similar infection response as humans during microbial pathogenesis. While research conducted with mammalian hosts is tedious, incurs mammalian suffering, and raises serious ethical issues, insects are suitable substitutes because of the similarities in insect and mammalian immunity and the commonalities in the infection processes. These congruences are underscored by the fact that

research utilizing insects such as *G. mellonella* and *D. melanogaster* for the study of opportunistic human pathogens, including strain virulence testing and the elucidation of putative virulence factors, show results are consistent with tests using mammalian hosts. A logical conclusion of these observations is that the mechanisms and essential evolutionary steps observed during the emergence of virulent insect pathogens may be similar to those required for the evolution of novel human pathogens. Thus, experimental evolution utilizing a model insect-pathogen system may elucidate general mechanisms of pathogen adaptation and the evolution of virulence that can be applied to novel emerging human diseases.

CONCLUSION

An understanding of pathogen evolution is essential in order to prevent the emergence of novel human diseases. In addition to the information gleaned from the inferences of the evolutionary pasts of current human pathogens, an experimental pathogen-host model system will undoubtedly yield unique insights. Using a model system, the features of pathogen evolution highlighted by data on the ancestry of known pathogens, including horizontal virulence gene transfer, de novo mutation and host association, and genome decay, could be empirically evaluated. *A. flavus* infections in *G. mellonella* larvae represent an ideal model system to study the evolution of an opportunistic pathogen with respect to a single animal host. As a pathogen, *A. flavus* is pathogenically well-characterized and genetically malleable, and its status as an opportunistic pathogen provides the potential for an increase in virulence, indicative of evolution, as it adapts to the host. Furthermore, this fungal-insect model system is valid for the study of pathogen evolution with application to emerging human diseases because the infection cycle follows the same stages as mammalian pathogenesis and the response of insects to pathogens is comparable to that of mammals.

Accordingly, the studies presented in this thesis utilizing *A. flavus* infections in *G. mellonella* provided insightful knowledge concerning de novo mutations and host association, genome decay, and virulence genes within the context of pathogen evolution. Chapter 2 describes the serial passage of *A. flavus* through *G. mellonella* larvae for five generations, providing a foundation for the remaining chapters of the thesis. This experimental design mimicked the spread of disease through a susceptible host population, an ideal opportunity for pathogen evolution. While Chapter 2 notes phenotypic changes noted with host association, namely a decreased ability of the fungus to grow on artificial media following repeated infections of an insect host, Chapter 3 assesses de novo genetic alterations that occurred during serial propagation through *G. mellonella* larvae. Compared with serial passage on artificial media, propagation through insects imposed a genetic bottleneck on the *A. flavus* population

such that genetic diversity was refined. The bottleneck was not the outcome of positive selection since there was no concomitant increase in virulence or virulence factor production.

Chapter 4 describes a strain of *A. flavus* isolated during serial propagation through *G. mellonella* larvae with characteristics of an obligate insect pathogen. While this strain remained pathogenic with unaltered virulence toward insects, sporulating adequately on the cadavers, it ceased to infect and/or sporulate on various plant hosts and proved equally difficult to grow on artificial media. In this instance, host restriction was associated with cysteine/methionine auxotrophy due to a single gene mutation affecting the capacity of the sulfate assimilation pathway to reduce sulfate to sulfite. Hypothetically, host restriction could provide the selection pressure required for an increase in virulence that should accompany pathogen evolution. Because the phenotype of the insect-restricted strain of *A. flavus* alluded to genome decay, Chapter 5 details the attempt to isolate the mutated gene. Although the mutation was not identified, it did not reside in either the APS kinase or PAPS reductase genes whose products are part of the sulfate assimilation pathway.

In addition to host restriction, Chapter 6 discusses the enhanced protease production by the insect-restricted strain when grown on insect substrates. Presumably cysteine/methionine auxotrophy contributed to sulfur starvation, the physiological response of which was to secrete extracellular proteases to degrade proteinaceous nutrient sources containing sulfur amino acids. Because proteases act as virulence factors in insect pathogenesis (St. Leger 1995), the increased protease production afforded this slow-growing auxotroph a level of virulence and cuticular penetration equal to that of the wild-type, highlighting the evolution of an alternate pathogenic strategy adopted by the insect-restricted strain. Chapter 7 experimentally investigates horizontal gene transfer by transforming the insect-restricted strain with the virulence factor Pr1, a protease, from the facultative insect pathogen *M. anisopliae*. Although successfully transformed, the recombinants expressed only low levels of Pr1 such that virulence did not increase. The study

emphasizes the necessity of an appropriate genetic background amenable to horizontal gene transfer.

Thus as anticipated, studies utilizing *A. flavus* infections in *G. mellonella* larvae provided valuable information regarding emerging disease, specifically with respect to de novo mutations accompanying host association, genome decay, and alterations in virulence factor production as aspects of pathogen evolution.

Chapter II:

Serial passage of the opportunistic pathogen *Aspergillus flavus* through an insect host yields decreased saprobic capacity

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Abstract

To study the early stages of the effect of host restriction on pathogen evolution, we subjected the opportunistic fungus *Aspergillus flavus* to a serial propagation scheme whereby insect-virulent conidia were selected for repeated passage through an insect host, *Galleria mellonella* larvae, for 5 generations. Of the 35 lineages promulgated through this scheme, there were no consistent changes in virulence, which was measured by percent mortality of the larvae. There were, however, increases in the number of conidia on the insect cadavers (9 of 35 lineages) and decreases in the number of days between death and the appearance of fungal growth on the cadavers (4 of 35 lineages). Notably, most of the lineages (28 of 35 lineages) demonstrated a statistically significant decrease in the diameter of the colonies subcultured onto artificial media, indicating a decreased ability to grow saprobically. Conversely, most of the *A. flavus* cultures successively grown on agar media (9 of 10 lineages) exhibited no change in colony diameter after 15 rounds of subculturing. Propagation of the opportunist *A. flavus* through the insect host *G. mellonella* resulted in a diminished capacity to grow on an alternate substrate, while maintaining or increasing its ability to use the host as a nutrient supply.

Introduction

Despite the development of effective control measures towards many known infectious diseases, the emergence of novel, highly virulent microorganisms is a considerable threat to agriculture, animal husbandry, and human health. It is postulated that facultative or obligate pathogens, defined as pathogens that efficiently infect and produce disease in healthy hosts either with (facultative) or without (obligate) the ability to replicate outside the host, evolved from opportunistic pathogens that thrive saprobically and afflict a broad range of immunocompromised hosts, although with low virulence (Scheffer 1991). Repeated host-pathogen association is undoubtedly a key factor in pathogen evolution as it provides the selection pressure required for the establishment of virulent phenotypes.

To study this potential factor in emerging diseases, we have adopted a fungal-insect model of infection whereby the opportunistic fungus *Aspergillus flavus* was serially propagated through an insect host, larvae of the greater wax moth, *Galleria mellonella*. This system represents the continuous propagation of an infectious microbe through a susceptible host, mimicking the effect, for example, of disease dissemination through an agricultural monoculture or continuous transmission of nosocomial infections in hospital wards. *A. flavus* secretes a broad range of enzymes that allow it to exploit a variety of nutrient sources, both as a saprobe and as an opportunistic pathogen, albeit with low virulence (St. Leger et al. 2000, St. Leger et al. 1997). *A. flavus* is capable of infecting and killing a variety of insects, including silkworms, caterpillars, lace bugs, and cockroaches (Gupta & Gopal 2002). Insect pathogenesis involves conidium germination on the surface of the insect cuticle followed by penetration through the cuticle to gain access to the nutrient-rich hemocoel. Ramification of fungal growth throughout the insect hemocoel results in insect death and subsequent conidial production (i.e. transmissible propagules) on the moribund host (Kumar et al. 2004). Both its status as an opportunistic pathogen and its broad range of substrate-degrading enzymes make *A. flavus* a model pathogen for the investigation of host adaptation and pathogen evolution. The larvae of *G. mellonella*

were chosen as the insect host for this study because they are easily manipulated to provide large numbers of susceptible hosts for pathogen propagation.

During this serial propagation scheme, we monitored various parameters of *A. flavus* infection of *G. mellonella* in order to detect changes in virulence and the ability to use either the insect or artificial media (potato dextrose agar) as a nutrient source. These data provide insight into the evolution of an opportunistic pathogen with respect to pathogenesis when restricted to a population of a single type of susceptible host and establish *A. flavus* infections in *G. mellonella* as an appropriate model for the study of emerging diseases.

Materials and Methods

Insect, fungal strains, and culture conditions

G. mellonella larvae were purchased from Peterborough Live Bait (Peterborough, Ontario). *A. flavus* strains 9308 and 6982 were obtained from the University of Alberta Microfungus Collection and Herbarium (Edmonton, Alberta). Fungal strains were maintained on 10ml volumes of potato dextrose agar (PDA) (Difco) incubated at 30°C for 7 d. Conidial suspension were prepared by using a sterile glass rod to dislodge conidia from a Petri dish into 0.01% Triton X-100. The suspensions were filtered through a glass funnel containing glass wool to remove mycelia.

Serial passage through *G. mellonella* larvae

Successive passage of *A. flavus* through *G. mellonella* larvae was performed using two infection methods: topical application and injection. For topical infection, individual larvae were inoculated on the dorsal surface with 5µl of a conidial suspension, and placed separately into clean plastic vials. The larvae were incubated at 30°C and monitored daily. To remove non-infective propagules from the insect cuticle, the larvae were surface sterilized on the day that they died in 1% NaOCl for 1 minute, followed by two 2-min washings in sterile water. (*A. flavus* failed to grow on previously frozen, dead larvae inoculated and surface sterilized in this manner, proving that 1% NaOCl effectively removed surface conidia from the insect cadavers.)

Cadavers were then placed individually in clean vials with a piece of sterile, moist tissue and incubated at 30°C to observe conidial growth on the insect cadaver. Topical application bioassays with concentrations of 1×10^8 , 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , and 1×10^3 conidia ml^{-1} showed that the minimum concentration required to give a statistically significant percent mortality by day 15 compared to the control using Chi-square tests was 1×10^5 conidia ml^{-1} ($\chi^2 = 8.32$; $p < 0.01$) (Figure 2-1a). Thus, all serial propagation lineages involving topical application used $5 \mu\text{l}$ of 1×10^5 conidia ml^{-1} . For insect infection by injection, $3 \mu\text{l}$ of a conidial suspension was injected into the hemocoel of each insect using a clean syringe (gauge = 22s). Insects were placed individually in clean plastic vials, incubated at 30°C, and monitored daily until death and subsequent conidiation on the insect cadaver occurred. A moist, sterile tissue was added when death occurred. A concentration of 4×10^4 conidia ml^{-1} was the minimum amount required to infect via injection ($\chi^2 = 4.31$; $p < 0.05$) as determined by injection assays with the concentrations of 1×10^5 , 7×10^4 , 4×10^4 , 1×10^4 , and 1×10^3 conidia ml^{-1} (Figure 2-1b). Consequently, all the serial propagation lineages involving infection by injection utilized $3 \mu\text{l}$ of 4×10^4 conidia ml^{-1} .

Serial propagation of *A. flavus* through the larvae of *G. mellonella* was performed by inoculating 25 larvae either topically or by injection and collecting the conidia from the first insect to die after day 1 and to subsequently sporulate. This dead insect was vortexed in 3ml of a 0.01% Triton X-100 (v/v) -- 0.02% chloramphenicol (w/v) solution. The insect cadaver was removed by filtration and the conidial suspension was quantified microscopically using a hemacytometer. This conidial suspension was used to inoculate 25 larvae, representing the next generation of fungal propagation through the insect. Fungal samples from each generation of each lineage were archived by mixing with an equal volume of 50% glycerol and storing at -80°C. In addition, a toothpick was used to point-inoculate the center of a Petri dish containing a 10-ml volume of Potato Dextrose Agar (PDA) (Difco) with conidia from each generation of

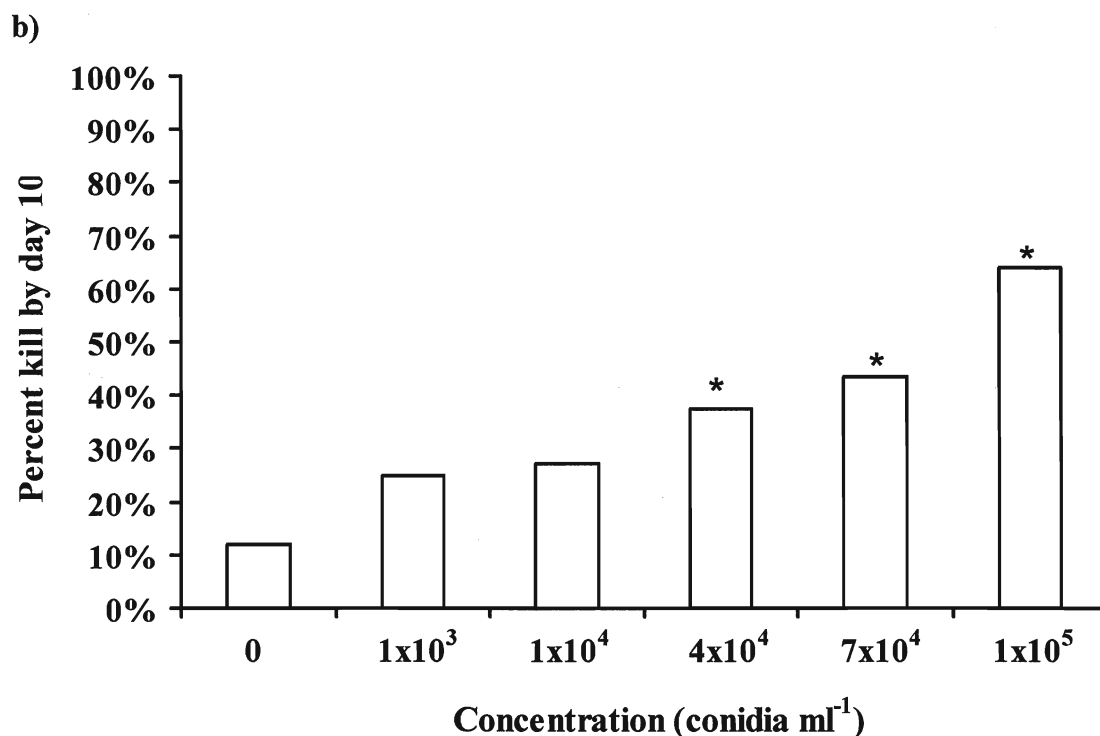
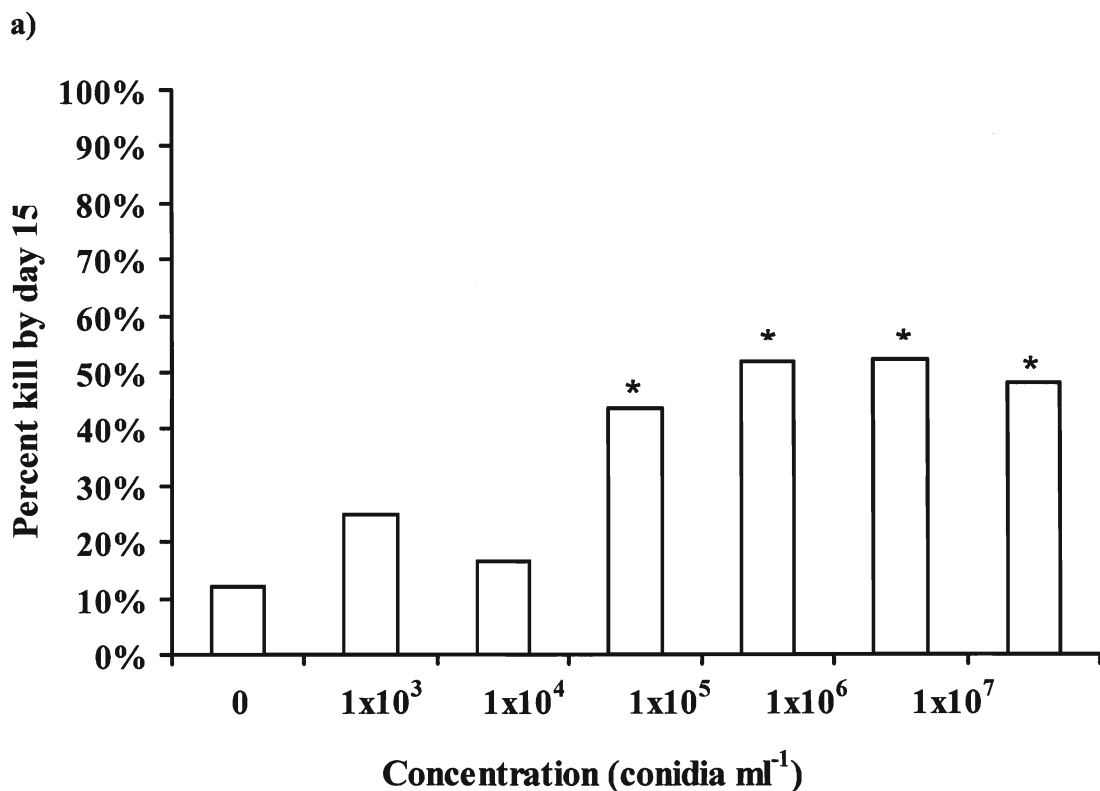


Figure 2-1: Concentration of *A. flavus* conidia required to give a statistically significant percent mortality by day 15 or day 10 respectively during (a) topical application (5 μ l) or (b) injection (3 μ l) infection of *G. mellonella* larvae incubated at 30°C. * indicates a statistically significant percent mortality compared to a control inoculation with 0.01% Triton X-100 (χ^2 test; $p < 0.05$).

passage through the insect. These subcultures were performed in triplicate and were incubated at 27°C for 7 d.

This scheme was repeated 35 separate times, creating 35 lineages of serial propagation of *A. flavus* through *G. mellonella*. Each lineage consisted of 5 generations of fungal passage through the host, with each generation enduring approximately 21 days. The 35 lineages were as follows: 5 lineages with *A. flavus* 9308 topical application, 5 lineages with *A. flavus* 6982 topical application, 5 lineages of double infection with equal amounts of *A. flavus* 9308 and *A. flavus* 6982 topical application, 5 lineages of mutated *A. flavus* 9308 topical application, 5 lineages of mutated *A. flavus* 6982 topical application, two lineages of *A. flavus* 9308 injection, two lineages of *A. flavus* 6982 injection, two lineages of double infection with equal amounts of *A. flavus* 9308 and *A. flavus* 6982 injection, two lineages of mutated *A. flavus* 9308 injection, and two lineages of mutated *A. flavus* 6982 injection. In the four sets of lineages designated “mutated,” the initial conidial suspension was mutagenized by exposure to UV-A light (400 $\mu\text{W}/\text{cm}^2$) for 3 min, which resulted in 80% conidial mortality, to augment the amount of initial genetic variability (see Appendix A).

The parameters monitored throughout this scheme were percent mortality by day 15 after topical infection (day 10 for injection), number of days between insect mortality and visible sporulation on the insect cadaver, diameter of the colonies subcultured onto a 10-ml-PDA plate incubated at 27°C for 7 d, and the number of conidia on the cadaver of the first insect to die after day 1. Percent mortality was assessed using the minimum dose required for significant difference from control mortality (5 μl of 1×10^5 conidia ml^{-1} for topical application and 3 μl of 4×10^4 conidia ml^{-1} for injection). Generally, facultative insect pathogens such as *Beauveria bassiana* and *Metarhizium flavoviridae* exhibit a sigmoidal dose-mortality response (Nowierski et al. 1996). As an opportunistic pathogen, *A. flavus* exhibits relatively low virulence toward *G. mellonella* larvae regardless of dose such that the dose-mortality sigmoidal response is flattened. Consequently, increasing the infection dose even to the maximum dose tested (5 μl of 1×10^8

conidia ml⁻¹ for topical infection or 3µl or 1x10⁵ for injection) did not significantly increase percent mortality above that obtained with the minimum dose ($p > 0.99$, topical application, χ^2 test; $p > 0.08$, injection, χ^2 test) (Figure 2-1). Thus, percent mortality was adequately assessed at each generation using the minimum dose stated above, which was also the dose used for serial propagation. When quantifying the amount of conidia on the first insect cadaver, the length of incubation between the first appearance of conidia on the cadaver and quantification was 5 d for the topical application lineages and 3 d for the injection lineages. These 4 parameters were subjected to linear regression analysis in order to detect changes in generation 1 through to generation 5.

Furthermore, conidia from the subculture from generation 5 of 2 of the lineages (lineage 5 of mutated *A. flavus* 9308 topical application; lineage 1 of mutated *A. flavus* 6982 topical application) were subsequently serially subcultured 3 times on PDA each incubated at 27°C for 7 d. Linear regression analysis was used to detect a change in colony diameter between the generation 5 subculture and the 3 subsequent PDA subcultures.

Correlation of colony diameter with fungal biomass

To show a direct correlation between colony diameter and fungal biomass, the subcultures from one of the selection lineages (lineage 5 of mutated *A. flavus* 9308 topical application) were removed from the agar media and weighed. The PDA Petri dishes containing the subcultures were individually immersed in boiling distilled water to liquefy the agar. The colony was then removed with a spoon, rinsed with hot distilled water to remove any remaining agar, placed in a preweighed dish, oven-dried at 55°C overnight, and weighed. The data was analyzed using linear regression.

Serial passage on PDA

A. flavus 9308 and *A. flavus* 6982 were subjected to 15 rounds of subculturing on PDA. Subcultures were created by using a sterile toothpick to transfer a small amount of fungal biomass from the outermost edge of a subculture to the center of a new PDA Petri dish. All

subcultures were incubated at 27°C for 7 d. The subculturing scheme was conducted 10 times to create 5 lineages of *A. flavus* 9308 and 5 lineages of *A. flavus* 6982. The colony diameters of the successive subcultures were measured and assessed with linear regression analyses.

Results

During the serial propagation of *A. flavus* through *G. mellonella* larvae, the topical application lineages and the injection lineages demonstrated similar responses in each of the 4 monitored parameters (Table 2-1; see Appendix B). The lineages showed no consistent changes in percent mortality, signifying no alteration in virulence (Figure 2-2; see Appendix B). Although none of the 10 injection lineages showed any statistically significant change in time to conidiation after death or number of conidia on the insect cadaver, a significant decrease in time to conidiation after death was noted in 4 of 25 topical application lineages (Figure 2-3; see Appendix B). Furthermore, 9 of the 25 topical application lineages demonstrated a statistically significant increase in the number of conidia on the insect cadaver, while 3 showed a decrease (Figure 2-4; see Appendix B). The statistically significant changes in these parameters were distributed throughout the lineages, indicating no correlation between a particular strain or starting condition (i.e. double infection or mutation) and the parameters that showed significant changes.

By far the most striking change that occurred during the serial propagation scheme was the significant decrease in subculture-colony diameter exhibited by 22 of the 25 topical application lineages and 6 of the 10 injection lineages following repeated propagation in an insect (Table 2-1; Figure 2-5; see Appendix B). In 2 of the lineages, conidia from the subculture from generation 5 was subsequently serially subcultured 3 times on PDA, and colony diameter did not increase (linear regression $F = 1.498, p = 0.252$; $F = 0.101, p = 0.758$). Colony diameter was directly correlated with fungal biomass, as indicated by a statistically significant increase in fungal biomass with colony diameter (linear regression $F = 23.36; p < 0.001$) (Figure 2-6).

However, when *A. flavus* strains 9308 and 6982 were serially propagated on PDA, the majority

Table 2-1: Summary data of linear regression analyses showing the proportion of lineages that show a significant ($p < 0.05$) increase or decrease in 4 parameters (percent mortality, time to conidiation after death, number of conidia on insect cadaver, and subculture-colony diameter) detected during the serial propagation by either topical application or injection of *A. flavus* through *G. mellonella* larvae for 5 generations (see Appendix B).

	Percent mortality		Time to conidiation after death		Number of conidia On insect cadaver		Subculture-colony diameter	
	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase
Top. Infect.	0/25	1/25	4/25	0/25	3/25	9/25	22/25	0/25
Injection	0/10	0/10	0/10	0/10	0/10	0/10	6/10	0/10

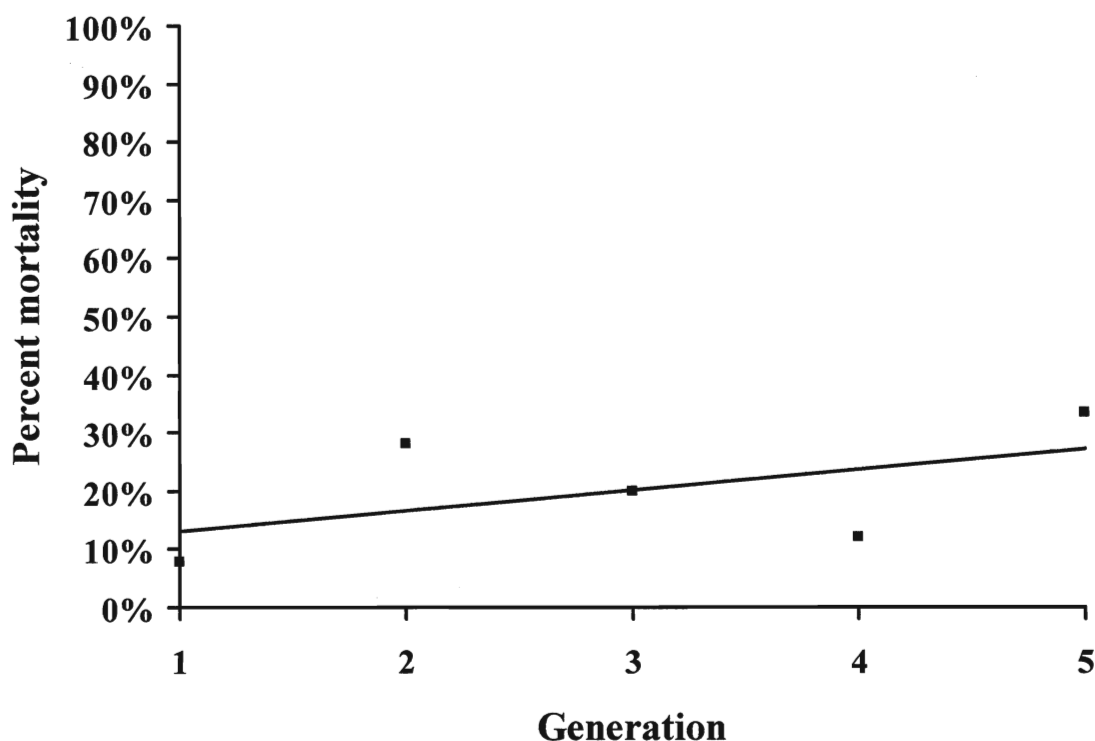


Figure 2-2: Linear regression of the percent mortality of *G. mellonella* larvae during the serial propagation of *A. flavus* through the larvae for 5 generations. Larvae were either topically infected or injected with *A. flavus* during the serial propagation scheme in which insect-virulent conidia were selected for subsequent serial passage through *G. mellonella*. One topical application lineage demonstrated a statistically significant increase. The remaining 24 topical application and 10 injection lineages demonstrated no significant change (data not shown) (see Appendix B).

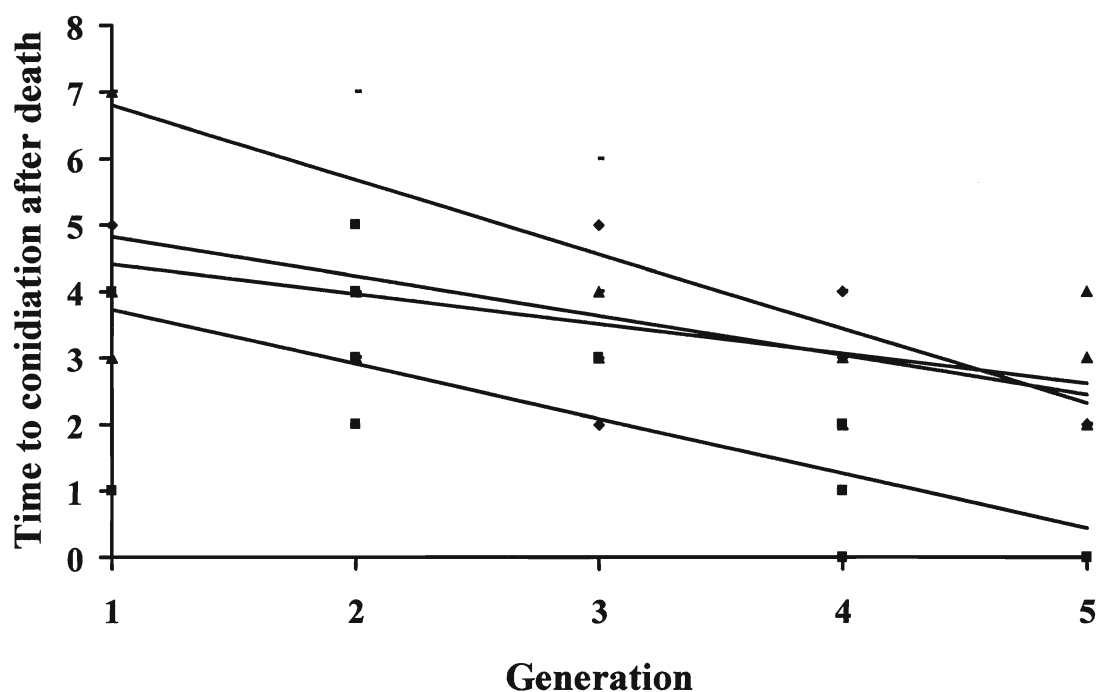


Figure 2-3: Linear regression of the number of days between the death of *G. mellonella* larvae and the conidiation of *A. flavus* on the cadavers over generations 1-5. Larvae were either topically infected or injected with *A. flavus* during the serial propagation scheme in which insect-virulent conidia were selected for subsequent serial passage through *G. mellonella*. Four topical application lineages showed a statistically significant decrease in the number of days between death and conidiation, while 21 topical application lineages and all 10 injection lineages demonstrated no significant change (data not shown) (see Appendix B).

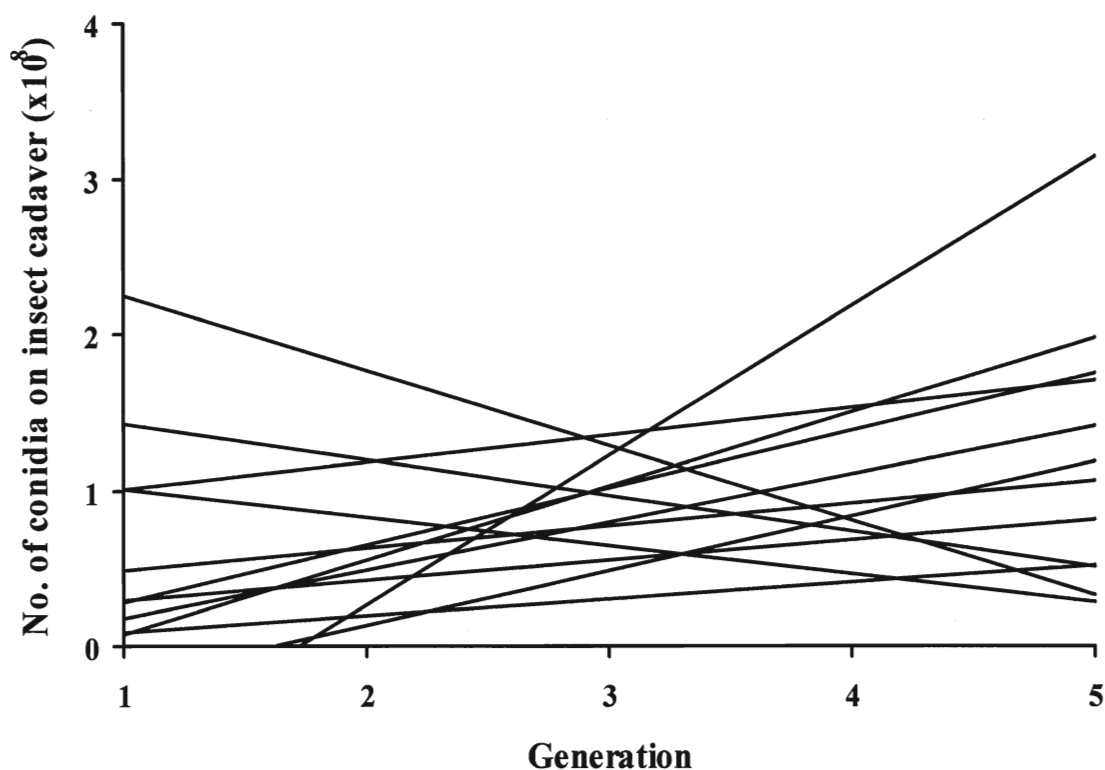


Figure 2-4: Linear regression of the number of *A. flavus* conidia on the first insect to die and conidiate over generations 1-5 of *G. mellonella* larvae. Larvae were either topically infected or injected with *A. flavus* during the serial propagation scheme in which insect-virulent conidia were selected for subsequent serial passage through *G. mellonella*. Nine topical applications lineages showed a statistically significant increase in the number of conidia on the cadaver while 3 topical application lineages showed a statistical decrease. Thirteen topical application lineages and 10 injection lineages demonstrated no significant change (data not shown) (see Appendix B).

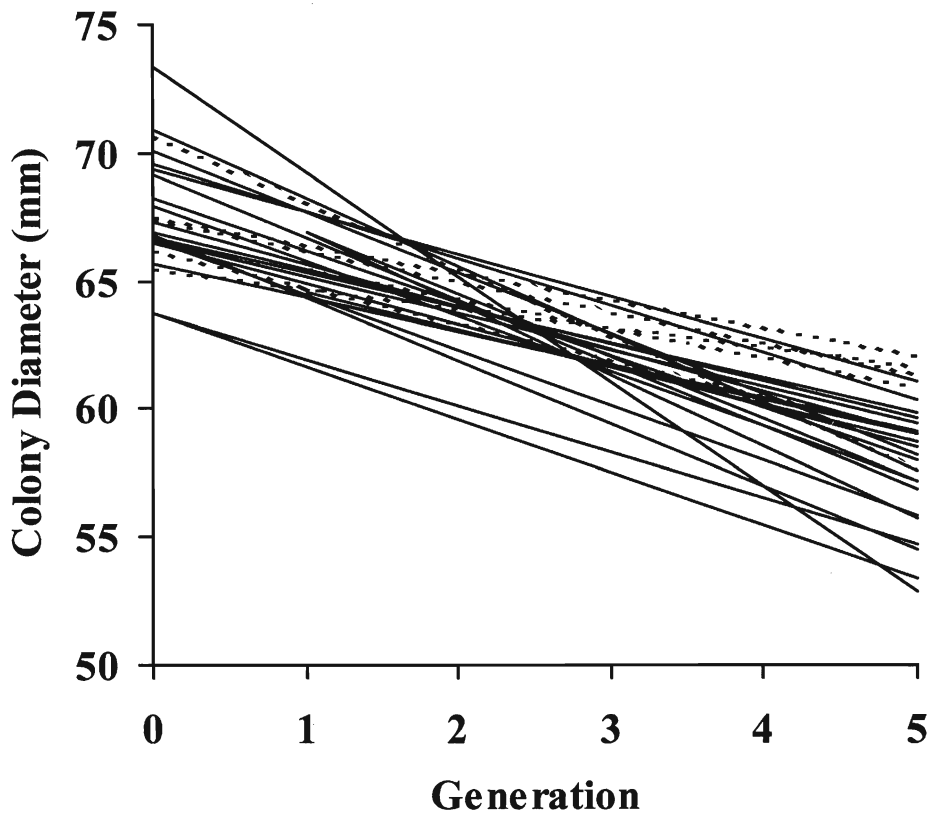


Figure 2-5: Linear regression of the colony diameter of the *A. flavus* subcultures from the first insect to die and conidiate over generations 0-5 of *G. mellonella* larvae. Larvae were either topically infected or injected with *A. flavus* during the serial propagation scheme in which insect-virulent conidia were selected for subsequent serial passage through *G. mellonella*. Twenty-two topical application lineages (solid lines) and 6 injection lineages (dashed lines) showed a statistically significant decrease in colony diameter, and 7 lineages in total demonstrated no significant change (data not shown) (see Appendix B).

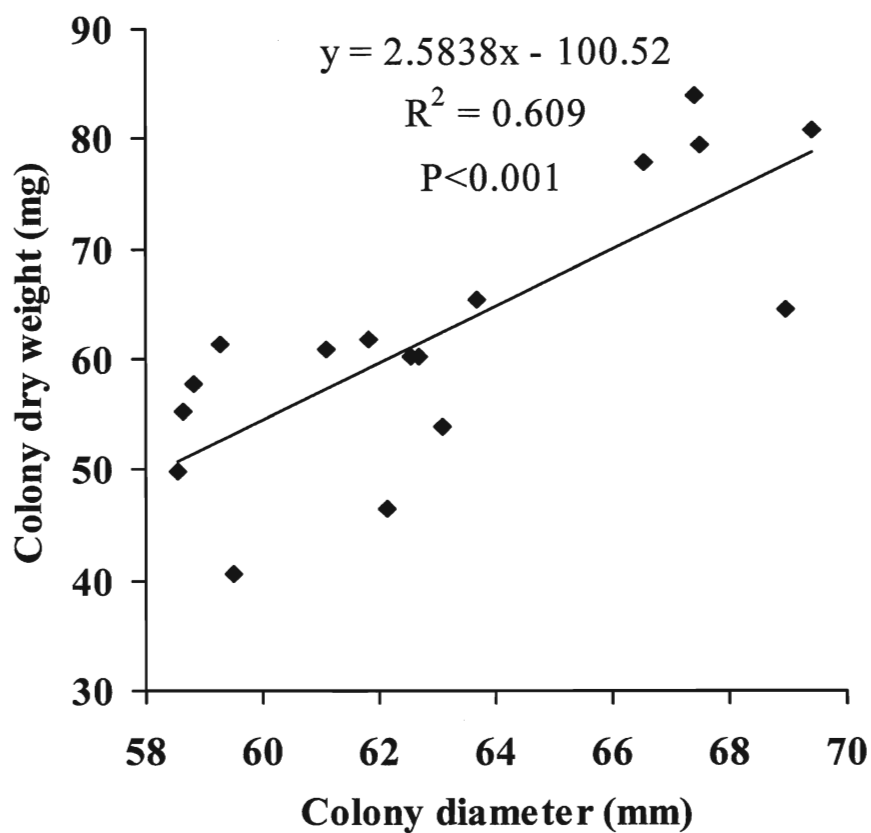


Figure 2-6: Correlation of fungal biomass with colony diameter of colonies grown on 10ml-PDA Petri dishes incubated at 27°C for 7 d. Data represent the subcultures of lineage 5 of mutated *A. flavus* 9038 topical application. There was a statistically significant increase in fungal biomass with colony diameter (linear regression $F = 23.36$, $p < 0.001$).

of the lineages (9 of 10) showed no statistically significant change in subculture-colony diameter (Figure 2-7, see Appendix C).

Discussion

When 35 lineages of *A. flavus* were serially propagated through *G. mellonella* larvae, only one of the lineages demonstrated an increase in virulence. However in 4 of the lineages, the time to conidiation after death decreased significantly, and in 9 lineages the amount of conidia on the insect cadaver increased significantly. These trends indicated a moderate adaptation by the fungus to aid its growth on the insect host and to utilize it as a nutrient source (Garber 1960). Surprisingly, 28 of the 35 lineages serially propagated through the insect showed a statistically significant decrease in subculture-colony diameter, which represented a significant decrease in fungal biomass on PDA. The smaller colony diameter was maintained upon further subculturing on PDA, indicating a heritable phenotypic change. The change was attributed to forced passage through the insect host and not due to the act of propagation itself, since serially subculturing *A. flavus* on PDA resulted in no change in colony diameter in majority of lineages. In essence, this signified a decrease in the ability of the fungus to grow as a saprobe on artificial media following forced restriction to the insect host. Thus, repeated restriction to the insect host resulted most consistently in a decreased ability to grow on an alternate nutrient source, while maintaining or possibly increasing its ability to utilize the insect as a nutrient source for the majority of lineages.

Serial passage experiments have been performed with various entomopathogenic fungi. While some of these studies involve an increase in virulence of entomopathogenic fungi during serial in vivo passage through an insect host, others do not. Serial in vivo passage experiments involving *Paecilomyces farinosus*, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Conidiobolus coronatus* demonstrated an increase in virulence (Hartmann & Wasti 1974; Hayden et al. 1992; Schaerffenberg 1964; Wasti & Hartmann 1975). However, similar studies with *Verticillium lecanii* and *Nomuraea rileyi* did not show significantly enhanced virulence after in vivo passage through an insect host (Hall 1980; Ignoffo et al. 1982). The lack of an

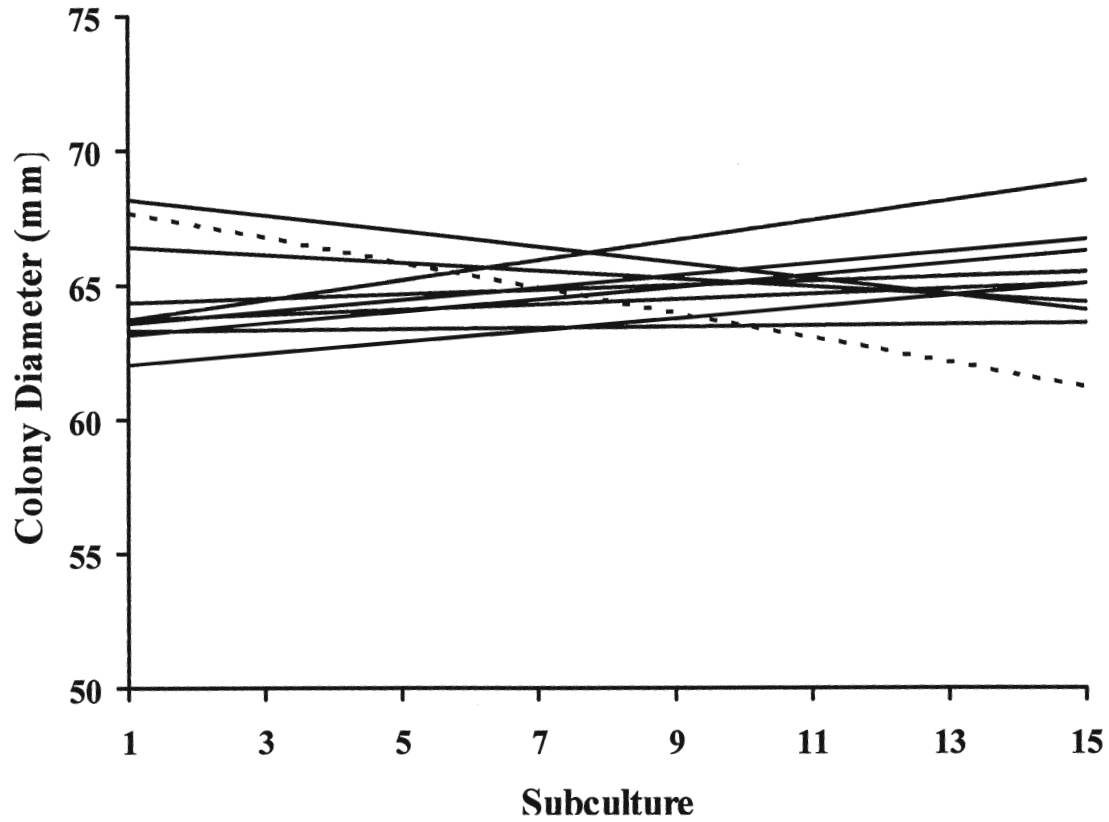


Figure 2-7: Linear regression of the colony diameter of *A. flavus* serially subcultured on PDA for 15 subcultures. One of the lineages (dashed lines) showed a statistically significant decrease in colony diameter, while 9 of the lineages (solid lines) demonstrated no significant change (see Appendix C).

increase in virulence observed in this study may represent differences between opportunistic pathogens such as *A. flavus* and the facultative insect pathogens mentioned above. Unlike the latter, the former may lack the genetic capacity for an increase in virulence selected for in the environment of the insect host.

Furthermore, serial passage experiments with pathogenic fungi typically demonstrate increased virulence, fitness, and growth on the host utilized for the passage accompanied by attenuation on the former host (Ebert 1998). For example, *Septoria nodorum* isolates are typically virulent to and produce growth only on either barley or wheat. Passage of a wheat biotype through barley resulted in the recovery of isolates that had shifted to the barley biotype, losing virulence toward and capacity to grow on wheat (Cunfer 1984). *Phytophthora infestans* propagated on a given potato variety increased in virulence and growth rate on that variety, while demonstrating attenuation on an alternate variety (Jinks & Grindle 1963). In a similar manner, the results of this study indicate a decreased ability of *A. flavus* to grow on an alternate nutrient source, while maintaining or augmenting its capacity to exploit the insect host as a nutrient supply (Garber 1960). In addition, a diminished ability to utilize an alternate nutrient source may precede an adaptation to an increase in virulence on a given host.

Concerning the emergence of novel infectious pathogens, the results of this study suggest that an increase in virulence by an opportunistic pathogen and the emergence of a novel facultative or obligate pathogen would be greatly facilitated by the acquisition of virulence factors through horizontal transmission. However, the constant association of an opportunistic pathogen with a given host may result in the adaptation of life history traits, such as a decreased propensity to grow saprobically, stimulating pathogen evolution and increases in virulence.

Chapter III:

The host acts as a genetic bottleneck during serial infections: An insect-fungal model system

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Abstract

The genetic variation of a pathogen population is a pivotal component of pathogen evolution, having important implications for emerging diseases, nosocomial infections, and laboratory subculturing practices. Furthermore, it is undoubtedly altered during infection of a host. We address this issue using an insect-fungal model system to examine the influence of serial host passage on the genetic variation of a pathogen population. Using amplified fragment length polymorphism, a strain of the opportunistic fungus, *Aspergillus flavus*, showing initially 98% genetic similarity, was assessed for changes in genetic diversity during repeated passage through *Galleria mellonella* larvae and compared to that of a parallel population serially subcultured on artificial media. In two independent trials, the genetic diversity of the population passed through the insect dropped significantly, while the genetic variation of the population subcultured on media increased or remained unchanged. However, there were no changes in virulence or the production of protease or aflatoxin, indicating an apparent lack of selection. We suggest that the insect acted as a genetic bottleneck, reducing the genetic diversity of the *A. flavus* population. The ability of a host to produce a genetic bottleneck in a pathogen population impacts our understanding of emerging diseases, nosocomial infections, and laboratory subculturing practices.

Introduction

The pressure the host exerts in shaping the genetic composition of an opportunistic pathogen population has implications for the study of emerging diseases, nosocomial infections, and laboratory culturing of microbial pathogens. Pathogen evolution and host response are interdependent as the host seeks to rid itself of the potential invader and the pathogen adapts to the complex and adverse environment, particularly in a novel, coincidental host. With respect to the emergence of a "new disease," the pathogen must initially have the ability to infect a relatively broad array of hosts but with little specialization. Following repeated infection of a new host, various evolutionary forces such as genetic bottlenecks and selection imposed by the host during the infection process can alter the genetic composition of the pathogen population (Souza et al. 2002). These changes in genetic composition can play a key role in microbial evolution contributing to the emergence of a virulent, novel, disease-causing pathogen. Thus, understanding the impact the host exerts on the genetic diversity of a pathogen population can further our understanding of pathogen evolution and the mechanisms by which novel diseases emerge.

Our research involves the experimental serial passage of the opportunistic fungal pathogen *Aspergillus flavus* through an insect host, wax moth (*Galleria mellonella*) larvae (Lepidoptera) (Scully & Bidochka 2005) as a model system for the study of disease emergence and pathogen evolution. Although much is known about the characteristics, virulence, and disease process of a variety of human and animal pathogens, little is known about the evolution and adaptation of disease-causing microbes. Consequently, we have adopted a fungal-insect model system as a simple, quick-cycling, easily-manipulated pathogen-host system for the study of pathogen evolution and adaptation to a host during repeated serial passage. *A. flavus* was chosen as the model pathogen in this system because it is an opportunistic pathogen capable of infecting a wide variety of hosts including plants, mammals, and insects (St. Leger et al. 2000). Although well-known as a saprophyte and a plant pathogen (Jones et al. 1980; Plasencia 2004),

A. flavus is capable of infecting lepidopteran larvae as a coincidental host via transcuticular penetration (Kumar et al. 2004), however, it is an inefficient insect pathogen and exhibits low virulence. Thus, in terms of an experimental system to investigate pathogen evolution, the opportunistic pathogen *A. flavus* demonstrates a potential for adaptation to this coincidental host.

In this study, we utilized this model system to examine the within-population genetic variation of an opportunistic pathogen population during successive passage through a coincidental host in order to assess the impact of the host on the genetic diversity of the pathogen population. We used amplified fragment length polymorphism (AFLP) fingerprints of multiple isolates of a single strain of *A. flavus* taken after various passages through wax moth larvae or following serial subculture on artificial media. The major finding was a significant drop in the genetic diversity below that of the initial population, while the genetic diversity during serial subculture either increased or was unaltered, and we argue that the drop in genetic diversity is a random genetic bottleneck rather than selection. Our findings model changes in genetic variation of a pathogen population during serial host passage and provides insight into pathogen genetic dynamics during nosocomial infections, emerging diseases, and laboratory culturing of microbial pathogens.

Materials and Methods

Serial propagation through the insect host and on artificial media

We performed the continuous propagation of *A. flavus* through wax moth larvae and on artificial media (potato dextrose agar, PDA). The insect infections and the serial passage of *A. flavus* through either the insect host or on PDA are previously described (Scully & Bidochka 2005). Briefly, *A. flavus* was serially propagated through the insect host by inoculating sets of 25 wax moth larvae (Peterborough Live Bait, Peterborough, Ontario) via topical application of conidia (500 conidia in 5 μ l) to individual larvae. The insects were incubated individually at 30°C and monitored to observe death and subsequent conidiation of the insect cadaver. Conidia from the first insect to die were collected by vortexing the cadaver in 0.01% Triton X-100,

archived at -80°C in 25% glycerol, and used to infect another set of 25 larvae. Seven sets of larvae were serially infected using this scheme, which was replicated to create multiple lineages. Replicate lineages of *A. flavus* were also serially subcultured on PDA by transferring a small piece of fungal material from the outside of a 7-day old colony incubated at 30°C to the center of a PDA plate using a sterile toothpick.

The initial strain of *A. flavus* 6982 (University of Alberta Microfungus Collection and Herbarium) used in the serial propagation schemes showed approximately 98% genetic similarity assessed via simple matching coefficients using AFLP data (see below) when received from the fungal collection. We did not originally isolate a single-conidium isolate since this allowed for the possibility to observe a decrease or an increase in genetic variation during passage through an insect or on an artificial medium. *A. flavus* 6982 was originally isolated as a contaminant on food for alfalfa leafcutting bee larvae.

The genetic variation of this strain during passage through either the insect host or on PDA was assessed by analyzing AFLP fingerprints (see below). AFLP fingerprints from 30 single conidium isolates each from subcultures 4 and 7 of serial passage through the wax moth larvae were evaluated. Sub_{4^{insect}} and Sub_{7^{insect}} indicate the resultant population after the fourth and seventh passages through the insect, respectively. Genetic variation during serial subculturing on artificial media (PDA) was also determined using AFLP fingerprints of 30 single conidium isolates each of subcultures 0, 4, and 7, representing the initial population (Sub_{0^{PDA}}) cultured on PDA and the population after the fourth subculture (Sub_{4^{PDA}}) and the seventh subculture (Sub_{7^{PDA}}) on PDA. Sub_{0^{PDA}} also represents the initial population cultured on PDA prior to serial passage through the insect. AFLP analyses of two independent trials were conducted and are designated lineages 1 and 2.

Isolate DNA preparation

Thirty isolates each of Sub_{0^{PDA}}, Sub_{4^{insect}}, Sub_{7^{insect}}, Sub_{4^{PDA}}, and Sub_{7^{PDA}} of lineages 1 and 2 were prepared by streaking samples from the suspension archived at --80°C onto PDA

using sterile cotton swabs. After incubation at 27°C for 3 d, single colonies were inoculated into flasks containing 50ml of YPD broth (0.2% yeast extract, 1.0% peptone, 2.0% dextrose) and grown at 25°C for 3 d at 200 rpm. Fungal material was removed via suction filtration and crushed in liquid nitrogen using a mortar and pestle. Genomic DNA was prepared using the DNeasy tissue extraction kit (Qiagen), yielding a total of 270 samples.

AFLP reactions

Five-hundred nanograms of fungal DNA was digested with 1 U EcoRI and 1 U MseI in a total volume of 10µl containing 100ng/µl BSA, 10mM Tris-HCl, 10mM MgCl₂, 50mM NaCl, 1mM DTT (pH 7.9 at 25°C) at 37°C for 16 h. Following digestion, reactions were incubated at 70°C for 15 min to stop the reaction. Ligation was performed in a total volume of 20µl by adding 2pmol of E adapter, 20pmol of M adapter (Table 3-1), and 0.4 U of T4 DNA ligase to the total volume of the restriction reaction. The ligation reactions were incubated at 20°C for 2 h and then at 4°C overnight. The restriction-ligation products were diluted 1:10 with TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) before amplification.

Pre-amplification was carried out in a total volume of 30µl, containing 5µl of diluted restriction-ligation product, 0.5µM primer EO, 0.5µM primer MO (Table 3-1), and 15µl of Taq PCR Master Mix (Qiagen). The PCR conditions for the pre-amplification were: 20 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s. The pre-amplification products were diluted 1:10 with TE.

Ten or 11 selective amplification reactions were performed using primers containing 1-3 selective nucleotides (Table 3-1). The primer combinations used were: EO/M+CAG, E+A/M+T, E+A/M+A, E+A/M+CAG, E+GC/MO, E+GC/M+T, E+GC/M+A, E+GC/M+CAG, E+AG/M+T, E+AG/M+A, E+AG/M+CAG. The selective amplification reactions contained 5µl diluted preamplification product, 0.5µM of each primer, and 11µl Taq PCR Master Mix (Qiagen) in a total volume of 22µl, and the PCR conditions were: 6 cycles of 94°C for 30 s, 65°C for 30 s,

Table 3-1: Adapters and primers used for AFLP analysis

Type	Name	Sequence
Adapter	E adapter	5'-CTCGTAGACTGCGTACC-3'
		3'-CATCTGACGCATGGTTAA-5'
	M adapter	5'-GACGATGAGTCC TGAG-3'
		3'-TACTCAGGACT CAT-5'
Primer	EO	5'-GACTGCGTACCAATTC-3'
	E+A	5'-GACTGCGTACCAATTCA-3'
	E+AG	5'-GACTGCGTACCAATTCAG-3'
	E+GC	5'-GACTGCGTACCAATTCGC-3'
	MO	5'-GATGAGTCCTGAGTAA-3'
	M+T	5'-GATGAGTCCTGAGTAAT-3'
	M+A	5'-GATGAGTCCTGAGTAAA-3'
	M+CAG	5'-GATGAGTCCTGAGTAACAG-3'

72°C for 60 s; 6 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s; 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s; followed by a final extension at 72°C for 5 min.

Ten or 11 AFLP fingerprints were performed for each of the *A. flavus* isolates. A total of 30 isolates were analyzed for each of the five subcultures. Furthermore, the serial propagation schemes were repeated, creating two lineages. Thus, a total of 2850 selective amplification reactions were performed and analyzed.

Polyacrylamide gel electrophoresis and silver staining

The selective amplification products were analyzed on 10% (19:1) denaturing polyacrylamide gels using a 38cm x 30cm x 0.75mm Sequi-Gen GT Nucleic Acid Electrophoresis Cell (BioRad). The gels were pre-electrophoresed at 70W to raise the temperature to 50°C. Samples were prepared by mixing 2µl of selective amplification product with 2µl of loading buffer (98% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol blue FF, 10mM EDTA) and denatured at 95°C for 2 min, followed by a rapid cooling to 4°C. The entire volume of each sample was loaded onto the gel and electrophoresed at 50°C for 2.5 h. Gels were silver-stained as described in Blum et al. (1987).

Data analysis

To assess the degree of genetic variation in Sub0_{PDA}, Sub4_{insect}, Sub7_{insect}, Sub4_{PDA}, and Sub7_{PDA} of lineages 1 and 2, the AFLP bands of 30 isolates of each subculture were scored binomially “1” for band presence and “0” for band absence. For each of the sets of isolates, a similarity matrix was constructed using simple matching coefficients based on the presence or absence of discrete AFLP bands (Sokal and Michener 1958) and subjected to cluster analysis by unweighted pair-group method using arithmetic averages (UPGMA) using NYSTS-pc version 2.1 (Exeter software 1986-2002).

Shannon’s informative index for phenotypic diversity was used to access genetic diversity based on AFLP fingerprints (Maughan et al. 1996; Zhu et al. 1998). The level of polymorphism is defined by:

$$H_s = -\sum f_i \ln(f_i)$$

where H_s is the phenotypic diversity value and f_i is the frequency of a given AFLP band in a set of isolates.

$$H_a = 1/n \sum H_s$$

is the average group diversity over n groups and

$$H_w = -\sum f \ln f$$

is the phenotypic diversity of the entire collection of isolates. The within-group and between-group diversities, H_a/H_w and $(H_w-H_a)/H_w$ respectively, were also compared. An analysis of the variance of the phenotypic diversity values obtained with the different primer pairs was assessed using the Kruskal-Wallis test (MINITAB).

Protease assays and aflatoxin production

Five isolates each of Sub0_{PDA}, Sub7_{insect}, and Sub7_{PDA} were individually assessed for subtilisin-like protease activity and trypsin-like protease activity during growth in either 1% cuticle media (prepared from *Melanoplus sp.* as described by Bidochka & Khachatourians (1990)) or synthetic *Galleria* media (SGM) (Dunphy & Webster 1986). Conidia from each isolate (1×10^5) were inoculated into 100ml YPD broth incubated at 25°C at 200rpm. After 3 d growth, the fungal material was filtered from the YPD, rinsed five times with sterile dH₂O to remove the YPD, and standard inocula (0.29-0.65g dry weight) were transferred to 25ml of either 1% cuticle or SGM. After 24 h of growth at 25°C and 200rpm, the extracellular filtrate was collected and filtered through a 0.22µm filter.

Enzyme assays were performed using nitroanalide (NA) substrates as described in St. Leger et al. (1987a). The reaction mixtures contained 180µl of 0.04M Tris buffer, pH 8.0 (subtilisin-like protease assay) or 0.04M Tris buffer, pH 8.5 (trypsin-like protease assay), 10µl of extracellular filtrate, and 10µl of 0.012M substrate. The substrate Suc-(Ala)₂-Pro-Phe-NA was used to detect subtilisin-like protease activity, while trypsin-like protease activity was monitored

using the substrate Bz-Phe-Val-Arg-NA. The reactions were followed at 410nm for 30 (trypsin-like) or 75 (subtilisin-like) min with results expressed as $\mu\text{mol NA released ml}^{-1} \text{ min}^{-1}$.

The five isolates of Sub0_{PDA}, Sub7_{insect}, and Sub7_{PDA} were assessed for aflatoxin production during growth in coconut extract broth (CEB) as described by Lemke et al. (1988).

Results

Polymorphisms detected by AFLP analysis

Using 10 or 11 primer combinations, 30 isolates each of Sub0_{PDA}, Sub4_{insect}, Sub7_{insect}, Sub4_{PDA}, and Sub7_{PDA} of lineages 1 and 2, yielding a total of 270 isolates, were evaluated for AFLP fragments. Figure 3-1 shows an example of the AFLP variation present among 30 isolates of Sub7_{PDA} of lineage 1 using a single primer pair (EO/M+CAG). In total, 590 fragments were detected in lineage 1 and 479 fragments in lineage 2 of which 82 (13.9%) and 44 (7.4%) were polymorphic, respectively (Tables 3-2 and 3-3). The number of fragments detected per primer pair ranged from 28 to 71 with an average of 53.5 for lineage 1 and 47.9 for lineage 2. On average 7.5 polymorphic fragments were detected per primer set for lineage 1 and 4.0 polymorphic fragments for lineage 2, with the number of polymorphic fragments detected per primer set varying between 1 and 13. The percentage of polymorphic fragments detected also varied from 1.7% to 46.4% depending on the number of selective nucleotides in the primers (Tables 3-2 and 3-3). Individual polymorphic fragments were present in a range of frequencies from 0.007 (present in only one isolate) to 0.993 (absent in only one isolate). In lineage 1, Sub7_{PDA} isolates showed the greatest number of polymorphic fragments (N = 51, 8.6%, Table 3-2), while Sub4_{insect} exhibited the least (N = 5, 0.8%, Table 3-2). Sub0_{PDA} isolates demonstrated the largest number of polymorphic fragments in lineage 2 (N = 15, 2.0%, Table 3-3), while Sub7_{insect} showed the smallest (N = 4, 0.5%, Table 3-3).

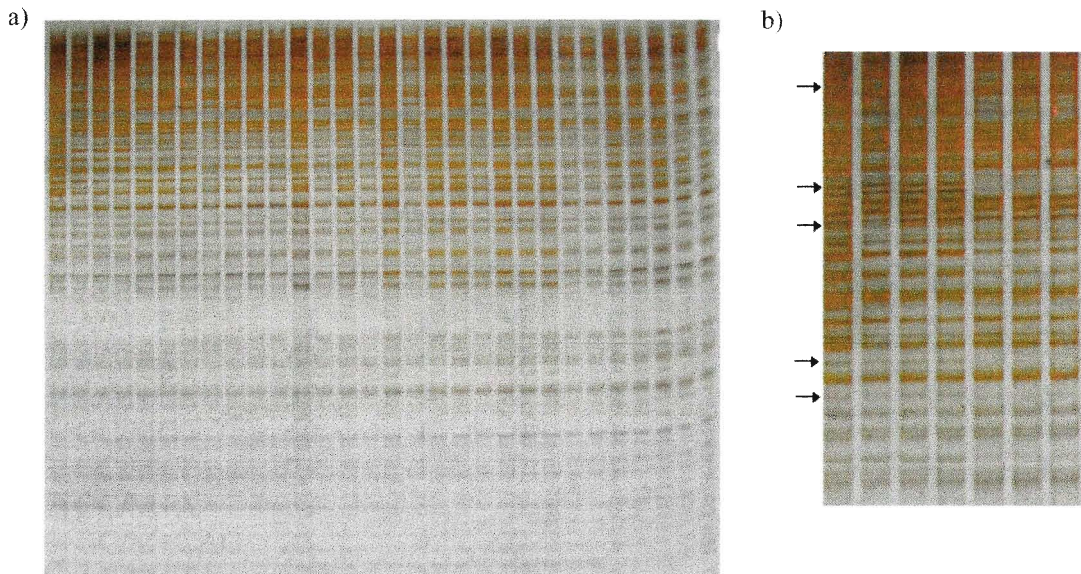


Figure 3-1: (a) Amplified fragment length polymorphism of 30 isolates of Sub7_{PDA} amplified using the primer pair EO/M+CAG. Selective PCR amplification products were separated on a denaturing polyacrylamide gel and silver stained. Fragments shown range from 1500bp-100bp. (b) Enlarged section of (a) where arrows indicate polymorphic fragments. Fragments shown range from 800bp-200bp.

Table 3-2: The number of AFLP bands and number of polymorphic bands detected in lineage 1 for Sub0_{PDA} (S0_{PDA}), Sub4_{insect} (S4_{insect}), Sub7_{insect} (S7_{insect}), Sub4_{PDA} (S4_{PDA}), and Sub7_{PDA} (S7_{PDA}) using various primer combinations.

Primer combination	No. of bands (N)	No. polymorphic bands detected (Np)					Shared	Total	Np/N
		S0 _{PDA}	S4 _{insect}	S7 _{insect}	S4 _{PDA}	S7 _{PDA}			
EO/M+CAG	58	1	0	0	1	7	0	9	15.5%
E+A/M+T	71	1	0	0	1	2	1	2	2.8%
E+A/M+A	64	1	0	1	1	5	1	5	7.8%
E+A/M+CAG	37	0	0	2	1	4	1	5	13.5%
E+GC/MO	50	1	0	1	1	2	1	4	8.0%
E+GC/M+T	71	1	0	1	1	7	1	8	11.3%
E+GC/M+A	59	3	3	0	3	4	2	9	15.3%
E+GC/M+CAG	33	4	7	3	2	5	4	16	33.3%
E+AG/M+T	62	1	0	0	2	5	1	7	11.3%
E+AG/M+A	60	0	1	0	3	7	1	10	16.7%
E+AG/M+CAG	28	3	0	7	4	3	4	13	46.4%
Total	590	16	5	15	20	51	17	82	
Average	53.6	1.5	0.5	1.4	1.8	4.6	1.5	7.5	
% Polymorphism		2.7%	0.8%	2.5%	3.4%	8.6%	2.9%	13.9%	

Table 3-3: The number of AFLP bands and number of polymorphic bands detected in lineage 2 for Sub0_{PDA} (S0_{PDA}), Sub4_{insect} (S4_{insect}), Sub7_{insect} (S7_{insect}), Sub4_{PDA} (S4_{PDA}), and Sub7_{PDA} (S7_{PDA}) using various primer combinations.

Primer combination	No. of bands (N)	No. polymorphic bands detected (Np)					Shared	Total	Np/N
		S0 _{PDA}	S4 _{insect}	S7 _{insect}	S4 _{PDA}	S7 _{PDA}			
EO/M+CAG	58	1	0	1	2	2	1	5	8.6%
E+A/M+T	59	1	0	0	0	0	0	1	1.7%
E+A/M+CAG	27	0	0	0	2	2	2	2	7.4%
E+GC/MO	51	1	0	0	0	2	0	3	5.9%
E+GC/M+A	60	3	1	2	0	0	0	6	10.0%
E+GC/M+T	55	1	3	1	0	0	1	4	7.3%
E+GC/M+CAG	28	4	1	0	1	1	0	7	25.0%
E+AG/M+T	57	1	0	0	5	1	0	7	12.3%
E+AG/M+A	54	0	0	0	0	1	0	1	1.9%
E+AG/M+CAG	30	3	4	0	0	2	1	8	26.7%
Total	479	15	9	4	10	11	5	44	
Average	47.9	1.5	0.9	0.4	1.0	1.1	0.5	4.0	
% Polymorphism		2.0%	1.2%	0.5%	1.3%	1.5%	0.8%	7.5%	

Cluster analysis

Similarity matrices were constructed for each of the five sets of isolates for lineages 1 and 2 using simple matching coefficients based on the presence or absence of the AFLP bands, and cluster analyses were performed according to the unweighted pair-group method using arithmetic averages (UPGMA). This provides an estimate of the degree of similarity between two individual isolates using the proportion of AFLP bands shared between them. In addition, it provides an estimate of the degree of genetic similarity based on the assumption that the same AFLP fragment patterns in two or more isolates represent the same genetic changes (Maughan et al. 1996). Among 150 isolates of lineage 1, the degree of similarity ranged from 0.916 to 1.000, while degree of similarity of lineage 2 isolates ranged from 0.972 to 1.000, indicating a high genetic similarity in both lineages regardless of propagation method. In lineage 1, Sub4_{insect} isolates demonstrated the highest level of similarity assessed by simple matching coefficients, as many of the isolates were rendered identical and all of the similarity coefficients were greater than 0.996 (Figure 3-2). In lineage 2, Sub7_{insect} isolates demonstrated the highest level of similarity with simple matching coefficients greater than 0.993 (Figure 3-3). In lineage 1, the Sub7_{PDA} isolates possessed the greatest dissimilarity between isolates as demonstrated by the cluster analysis with similarity coefficients as low as 0.921 (Figure 3-2), while the Sub0_{PDA} isolates exhibited the greatest dissimilarity for lineage 2 with values ranging from 1.000 to 0.980 (Figure 3-3).

Genetic diversity

The genetic diversity of the insect and PDA populations of lineages 1 and 2 were assessed by applying Shannon's information index for phenotypic diversity (Maughan et al. 1996; Zhu et al. 1998) to the AFLP fingerprints. In lineage 1, the phenotypic diversity based on the AFLP data of the entire collection of isolates (H_w) varied from 0.24 to 1.16 with an average score of 0.58 (Table 3-4). Sub7_{PDA} demonstrated the highest level of diversity with an average H_s of 1.04. The lowest diversity was exhibited by Sub4_{insect} with an average H_s of 0.03, while

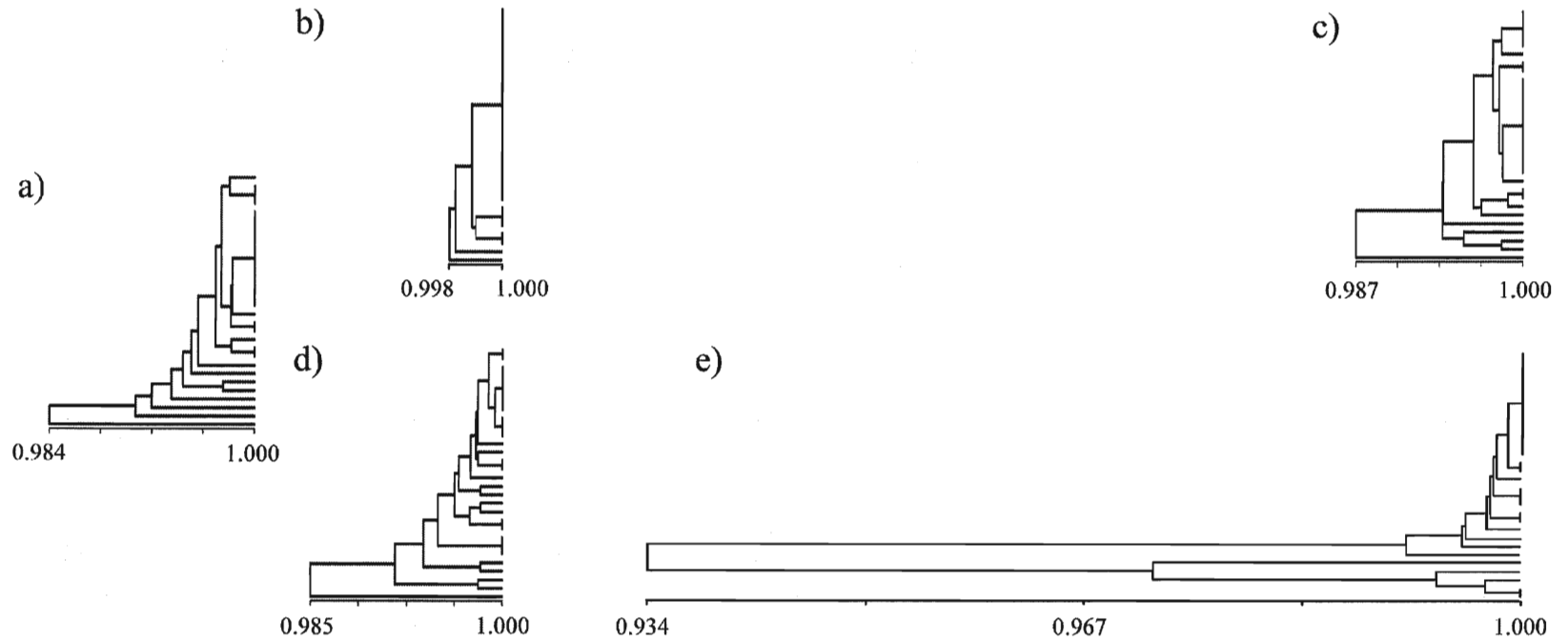


Figure 3-2: UPGMA cluster analyses derived from similarity matrices generated based on simple matching coefficients using the presence or absence of AFLP fragments in lineage 1. (a) Sub0_{PDA} (b) Sub4_{insect} (c) Sub7_{insect} (d) Sub4_{PDA} (e) Sub7_{PDA}. Scales represent simple matching coefficients.

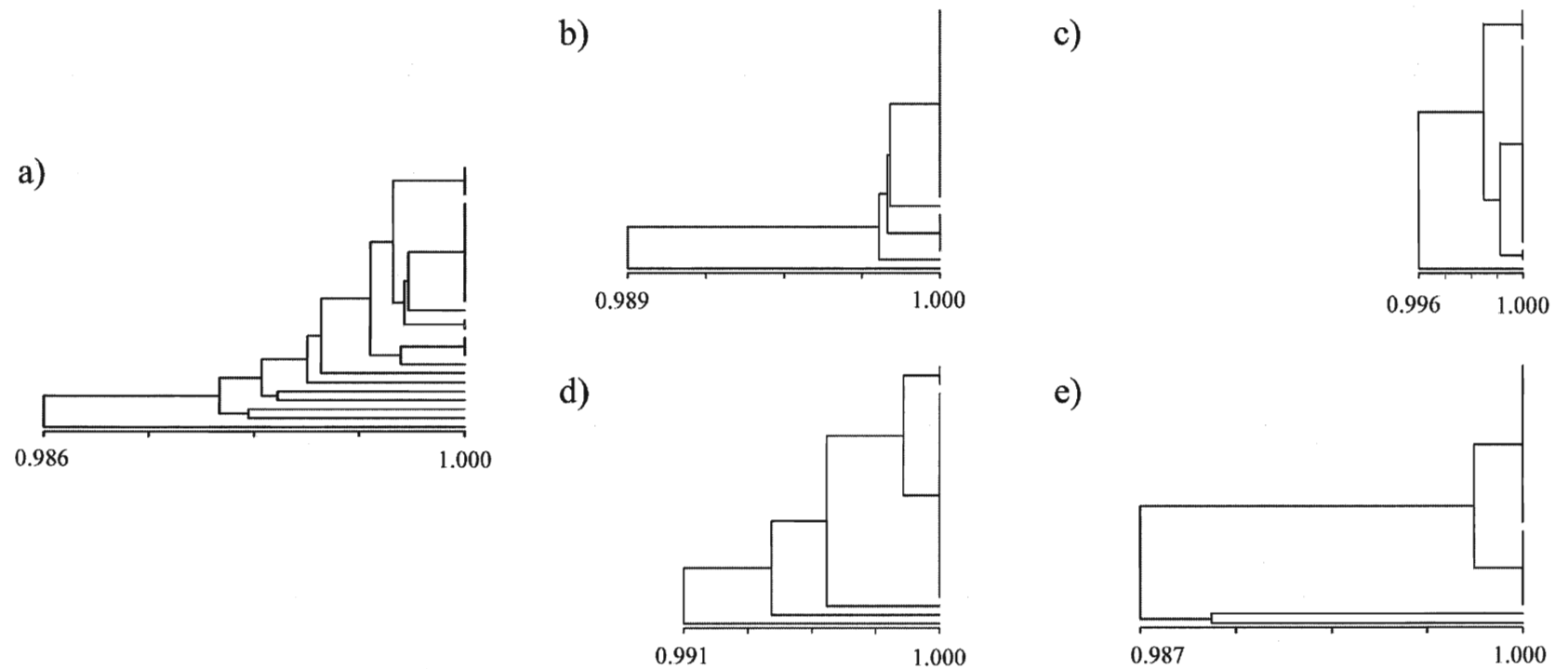


Figure 3-3: UPGMA cluster analyses derived from similarity matrices generated based on simple matching coefficients using the presence or absence of AFLP fragments in lineage 2. (a) Sub0_{PDA} (b) Sub4_{insect} (c) Sub7_{insect} (d) Sub4_{PDA} (e) Sub7_{PDA}. Scales represent simple matching coefficients.

Table 3-4: Genetic diversity of lineage 1 assessed for Sub0_{PDA} (S0_{PDA}), Sub4_{insect} (S4_{insect}), Sub7_{insect} (S7_{insect}), Sub4_{PDA} (S4_{PDA}), and Sub7_{PDA} (S7_{PDA}) using Shannon's information index for phenotypic diversity applied to AFLP fingerprints of 30 isolates from each of the groups using various primer combinations.

Primer combination	Diversity (H _s)					H _w	H-intra ^a	H-inter ^a
	S0 _{PDA}	S4 _{insect}	S7 _{insect}	S4 _{PDA}	S7 _{PDA}			
EO/M+CAG	0.13	0.00	0.00	0.18	1.61	0.65	0.59	0.41
E+A/M+T	0.11	0.00	0.00	0.19	0.61	0.28	0.66	0.34
E+A/M+A	0.28	0.00	0.24	0.35	1.25	0.82	0.52	0.48
E+A/M+CAG	0.00	0.00	0.43	0.23	1.19	0.61	0.61	0.39
E+GC/MO	0.04	0.00	0.12	0.12	0.49	0.24	0.61	0.39
E+GC/M+T	0.18	0.00	0.10	0.09	1.36	0.68	0.51	0.49
E+GC/M+A	0.19	0.12	0.00	0.25	0.52	0.31	0.71	0.29
E+GC/M+CAG	0.92	0.11	0.49	0.34	1.01	1.16	0.50	0.50
E+AG/M+T	0.19	0.00	0.00	0.39	1.35	0.66	0.58	0.42
E+AG/M+A	0.00	0.12	0.00	0.22	1.34	0.52	0.64	0.36
E+AG/M+CAG	0.29	0.00	0.24	0.37	0.73	0.48	0.68	0.32
Average	0.21	0.03	0.15	0.25	1.04	0.58	0.60	0.40

^aH-intra represents within-group diversity; H-inter represents between-group diversity

Sub0_{PDA}, Sub7_{insect}, and Sub4_{PDA} had intermediate average H_s values of 0.21, 0.15, and 0.25 respectively (Table 3-4). Kruskal-Wallis analyses of the individual H_s values for each sample revealed a significant distribution ($H = 42.80$; $p < 0.001$). The Sub7_{PDA} diversity values were significantly greater than those of Sub0_{PDA}, Sub4_{insect}, Sub7_{insect}, and Sub4_{PDA} ($p = 0.0001$, $p < 0.0001$, $p < 0.0001$, $p = 0.0060$ respectively). In addition, the H_s value for Sub4_{insect} was significantly lower than both Sub0_{PDA} ($p = 0.01997$) and Sub4_{PDA} ($p = 0.0007$). Sixty percent of the diversity of the AFLP patterns was due to intra-group variation, and 40% was due to inter-group variation (Table 3-4).

Lineage 2 demonstrated similar trends regarding diversity. The diversity of the 150 isolates (H_w) ranged from 0.01 to 0.46 depending on the selective amplification with an average of 0.17 (Table 3-5). Sub7_{insect} exhibited the lowest level of diversity with an average H_s of 0.02. The average diversity levels of Sub4_{PDA} ($H_s = 0.05$), Sub7_{PDA} ($H_s = 0.12$), and Sub4_{insect} ($H_s = 0.11$) were below that of Sub0_{PDA} ($H_s = 0.20$) (Table 3-5). Kruskal-Wallis analyses of the individual H_s values for each sample revealed a significant distribution ($H = 9.60$; $p < 0.048$). The genetic diversity value of Sub7_{insect} was significantly less than that of Sub0_{PDA} ($p = 0.0066$), while the remaining sets of isolates showed no statistically significant differences. AFLP patterns of lineage 2 were attributed 74% to intra-group variation and 26% inter-group variation.

The genetic diversity of both lineages of the *A. flavus* population demonstrated appreciable differences depending on whether the population was propagated as a pathogen through the insect host or saprobically on artificial media (Figure 3-4). In lineage 1, genetic diversity increased significantly in the population subjected to saprobic passage on artificial media compared to the population restricted to the insect host. The population after 4 passages through the insect exhibited a significantly lower genetic diversity than either the initial population or the population subcultured on artificial media. Furthermore, the Sub7_{PDA} isolates showed a significantly greater genetic diversity than all of the other sets of isolates (Figure 3-4a). In lineage 2, the population demonstrated a significant decrease in genetic diversity after the

Table 3-5: Genetic diversity of lineage 2 assessed for Sub0_{PDA} (S0_{PDA}), Sub4_{insect} (S4_{insect}), Sub7_{insect} (S7_{insect}), Sub4_{PDA} (S4_{PDA}), and Sub7_{PDA} (S7_{PDA}) using Shannon's information index for phenotypic diversity applied to AFLP fingerprints of 30 isolates from each of the groups using various primer combinations.

Primer combination	Diversity (H _s)							
	S0 _{PDA}	S4 _{insect}	S7 _{insect}	S4 _{PDA}	S7 _{PDA}	H _w	H-intra ^a	H-inter ^a
EO/M+CAG	0.13	0.00	0.04	0.18	0.23	0.15	0.76	0.24
E+A/M+T	0.11	0.00	0.00	0.00	0.00	0.03	0.66	0.34
E+A/M+CAG	0.00	0.00	0.00	0.07	0.07	0.03	1.00	0.00
E+GC/MO	0.04	0.00	0.00	0.00	0.38	0.12	0.67	0.33
E+GC/M+A	0.19	0.03	0.07	0.00	0.00	0.07	1.00	0.00
E+GC/M+T	0.18	0.53	0.10	0.00	0.00	0.44	0.37	0.63
E+GC/M+CAG	0.92	0.11	0.00	0.11	0.24	0.46	0.60	0.40
E+AG/M+T	0.19	0.00	0.00	0.16	0.18	0.15	0.71	0.29
E+AG/M+A	0.00	0.00	0.00	0.00	0.03	0.01	0.94	0.06
E+AG/M+CAG	0.29	0.46	0.00	0.00	0.10	0.24	0.72	0.28
Average	0.20	0.11	0.02	0.05	0.12	0.17	0.74	0.26

^aH-intra represents within-group diversity; H-inter represents between-group diversity

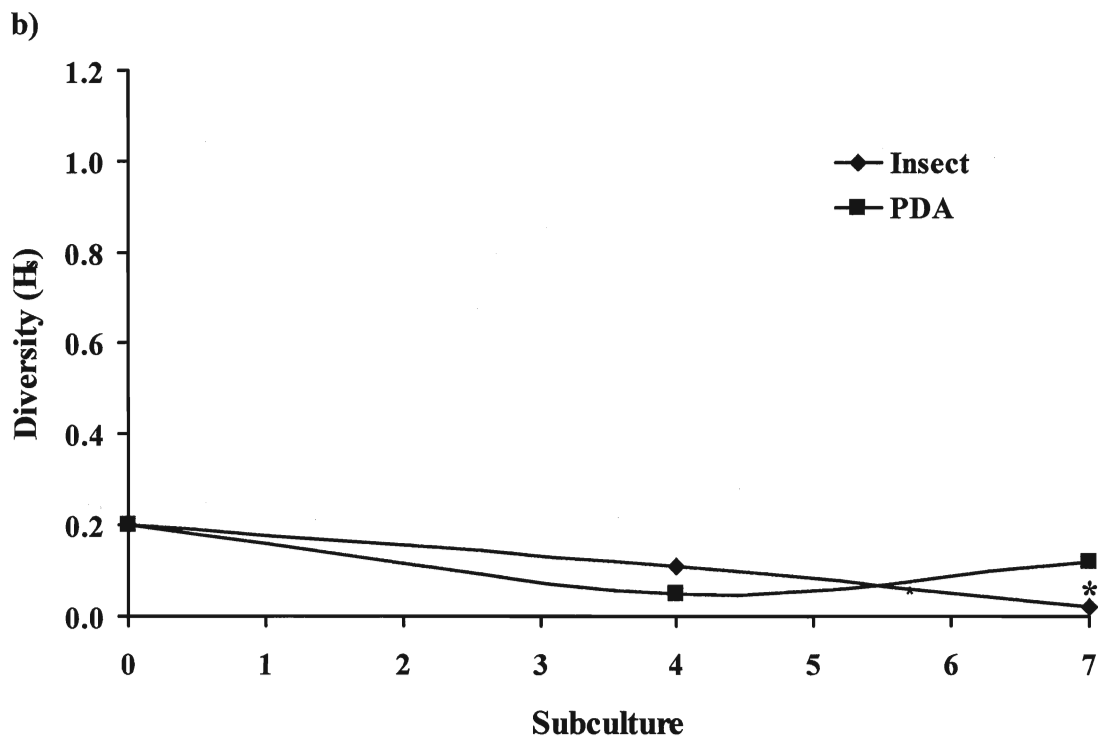
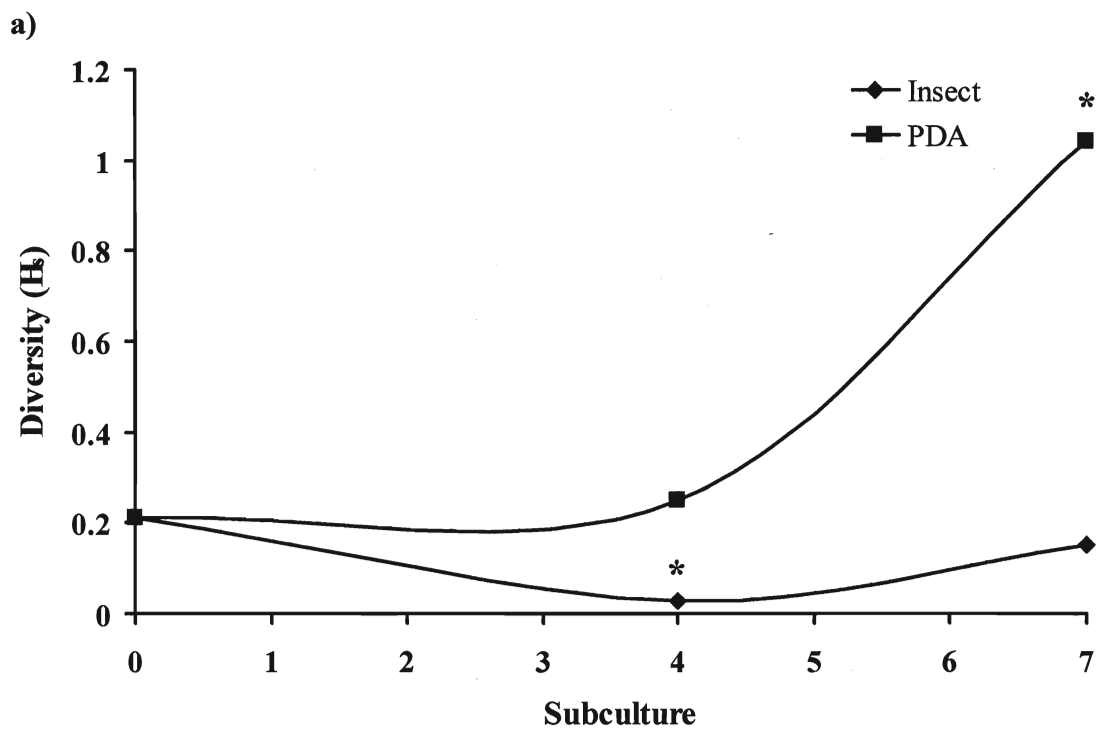


Figure 3-4: Assessment of genetic diversity derived from the average phenotypic diversity values resulting from AFLP data of isolates either serially passed through the insect host for seven generations or serially subcultured on PDA for seven subcultures. (a) Lineage 1 (b) Lineage 2 (* denotes statistical significance, family alpha = 0.2, Bonferroni individual alpha = 0.02).

seventh passage through the insect compared to the original population (Sub0_{PDA}), while the genetic diversity levels of lineage 2 propagated on artificial media remained statistically unchanged (Figure 3-4b).

Protease activity and aflatoxin production

Five isolates each of Sub0_{PDA}, Sub7_{insect}, and Sub7_{PDA} were assessed for subtilisin-like and trypsin-like protease activity in either 1% cuticle media or SGM. These media are intended to mimic the conditions experienced during infection of an insect either during transcuticular penetration at the initial stages of fungal insect pathogenesis (1% cuticle) or during the later stages of infection when fungi ramify through the hemocoel, killing the insect (SGM). The isolates produced more subtilisin-like and trypsin-like protease activity during growth in 1% cuticle media compared to SGM (Table 3-6). Kruskal-Wallis tests indicate that there were no significant differences between the three sets of isolates, Sub0, Sub7_{insect}, Sub7_{PDA}, for either type of protease activity in either growth media (subtilisin-like protease activity in 1% cuticle, $p = 0.059$; trypsin-like protease activity in 1% cuticle $p = 0.054$; subtilisin-like protease activity in SGM $p = 0.174$; trypsin-like protease activity in SGM $p = 0.075$).

None of the isolates of Sub0_{PDA}, Sub7_{insect}, or Sub7_{PDA} produced aflatoxin as assessed by the lack of fluorescence when grown in CEB.

Discussion

Changes in genetic variation of a pathogen population during host passage have important implications for understanding genetic dynamics during nosocomial infections, emerging diseases, and laboratory culturing of microbial pathogens. Using an insect-fungal model system, we have investigated the within-population genetic diversity of a population of an opportunistic pathogen (*A. flavus*) during serial passage through a host (wax moth larvae) to elucidate the influence that pathogenesis of a host has on the genetic diversity of a microbial population. The major finding was that genetic diversity, as measured by AFLP analyses, decreased significantly during passage through an insect host. Conversely, genetic diversity

Table 3-6: Extracellular subtilisin-like and trypsin-like protease activity of five isolates each of Sub0_{PDA}, Sub7_{insect}, and Sub7_{PDA} after 24h of growth in 1% cuticle media or SGM. Results are expressed as $\mu\text{mol NA ml}^{-1}\text{min}^{-1} \pm \text{S.E.} \times 10^6$. The experiment was repeated with similar results.

Pop.	Is.	Subtilisin-like activity		Trypsin-like activity		Aflatoxin
		1% Cuticle	SGM	1% Cuticle	SGM	
Sub0 _{PDA}	1	161.21 \pm 1.15	3.89 \pm 0.31	115.53 \pm 6.23	19.44 \pm 5.43	-
	2	74.80 \pm 5.66	2.98 \pm 0.40	135.86 \pm 10.42	41.79 \pm 11.96	-
	3	77.42 \pm 6.99	5.51 \pm 0.48	162.37 \pm 8.11	24.75 \pm 3.65	-
	4	87.27 \pm 2.28	4.75 \pm 0.10	133.33 \pm 14.39	30.43 \pm 5.71	-
	5	92.42 \pm 4.78	4.60 \pm 1.09	180.05 \pm 14.93	34.34 \pm 5.01	-
Sub7 _{insect}	1	83.13 \pm 7.79	4.24 \pm 0.32	152.78 \pm 9.23	18.94 \pm 2.30	-
	2	65.05 \pm 7.03	3.74 \pm 0.18	145.83 \pm 11.48	27.15 \pm 5.91	-
	3	96.46 \pm 8.30	2.93 \pm 0.79	190.78 \pm 14.84	33.33 \pm 9.31	-
	4	85.61 \pm 0.95	1.16 \pm 0.62	137.88 \pm 7.69	79.29 \pm 12.85	-
	5	87.58 \pm 5.59	1.87 \pm 0.27	157.45 \pm 7.30	81.44 \pm 9.22	-
Sub7 _{PDA}	1	90.61 \pm 5.76	1.21 \pm 0.70	188.26 \pm 7.13	43.56 \pm 3.22	-
	2	103.48 \pm 1.31	1.52 \pm 1.44	161.62 \pm 5.25	61.62 \pm 4.32	-
	3	104.65 \pm 2.01	4.09 \pm 2.05	171.84 \pm 24.51	93.69 \pm 8.49	-
	4	113.89 \pm 7.58	2.53 \pm 1.31	203.28 \pm 2.53	48.61 \pm 4.84	-
	5	86.57 \pm 7.85	12.17 \pm 11.11	199.87 \pm 20.23	59.22 \pm 10.85	-

during propagation on artificial media resulted in either a significant increase or no statistical difference in genetic variation compared to the initial population. A decrease in genetic diversity during passage through a host emphasizes the impact the host exerts in shaping the evolution of a pathogen population.

The two lineages of this study demonstrated appreciable differences in genetic diversity and makeup. Such differences highlight the random nature involved in mutations and genetic bottlenecks during serial passage also noted by Cowen et al. (2000) in replicate populations of *Candida albicans* propagated in the presence of fluconazole. As expected for asexually reproducing *A. flavus*, all of the 270 isolates analyzed in this study were highly similar with similarity indices greater than 0.918, which was also noted in AFLP analyses of multiple isolates of the same strain of other clonal microbes (Janssen et al. 1997; Savelkoul et al. 1999). As this experiment precludes the influx of gene flow through recombination with novel genotypes, high similarity between isolates is expected as any variation is presumably due to de novo mutation events.

The level of genetic diversity of the *A. flavus* population serially subcultured on PDA increased significantly (lineage 1) or remained statistically unchanged (lineage 2) when compared to the original population. Isolates from the *A. flavus* population propagated saprobically on artificial media experienced a less restrictive environment than those passed through the insect, presumably allowing for the survival of more genetic variants in that population. Similar trends were observed in *Chlamydomonas* propagated in light and dark environments. Greater genetic variation was observed when they were propagated in the light, which represented a less restrictive environment, than in the dark (Bell & Reboud 1997).

Experimental evolution with various microbes has resulted in genetic changes responsible for an adaptive response (Cowen et al. 2000; Dunham et al. 2002; Nakatsu et al. 1998; Papadopoulos et al. 1999; Schneider et al. 2000) as well as those that are neutral or only weakly adaptive (Cowen et al. 2000; Schneider et al. 2000). Although Souza et al. (2002) argues that the

host can act as a “selective sieve,” reducing genetic diversity by eliminating less-fit variants in a pathogen population, the variants of the insect propagation scheme were no better adapted to insect pathogenesis than the isolates of the serial subculturing scheme despite the genetic refinement. In addition to no increase in virulence, (Scully & Bidochka 2005), isolates of the insect propagation scheme showed no significant increase in protease production, a virulence factor known to instigate penetration of the insect cuticle during infection with facultative insect pathogens (St. Leger 1995) and no aflatoxin production known to be toxic to *Bombyx mori* (Ohtomo et al. 1975) during growth in media that mimic nutrient conditions during the initial (1% cuticle) or later (SGM) stages of insect pathogenesis. Because the isolates generated during passage through the insect failed to show adaptation towards insect pathogenesis through increased virulence or virulence factor production, the lack of genetic variation displayed by these isolates is probably due to a genetic bottleneck rather than the selective elimination of less fit variants. All of the *A. flavus* genetic variants may have equal ability to adhere to and infect the insect host, however, variations in the random nature of conidial attachment and penetration through varying thicknesses of the insect cuticle as well as the random probability of host avoidance mechanisms and re-emergence from the host to conidiate on the surface may all play a role in creating a genetic bottleneck.

Our results correlate well with other studies that note phenotypic and genotypic alterations that occur in clonal pathogens during maintenance on artificial media (Horn & Dorner 2001; Iguchi et al. 2002; Kamp & Bidochka 2002; Yamashita et al. 1996). Furthermore, our study also indicates that the decline in pathogen virulence and virulence factor production observed when microbes are repeatedly cultured outside the host (Brummer et al. 1990; Carrol & Gherardini 1996; Schaerffenberg 1964; Watt et al. 2003) may be due to the higher number of genetic variants, some of which may be less virulent or avirulent, that can survive during subculturing on artificial media. As a consequence, it is recommended that pathogens cultured in vitro be periodically passed through their host to maintain virulence (Schaerffenberg 1964),

presumably by eliminating non-pathogenic variants that may have arisen during serial transfer on artificial media.

Our observation of the genetic bottleneck of the *A. flavus* population passed through the insect host correlates well with the observation that the population structure of many pathogens implicated in nosocomial outbreaks is clonal. *Pseudomonas aeruginosa* isolates obtained from an outbreak in an intensive care unit in Italy (Luzzaro et al. 2001), opportunistic bacteria *Staphylococcus aureus* isolated during a worldwide survey of nosocomial epidemics (Aires de Sousa & de Lencastre 2004), and clinical isolates of the filamentous fungus *Fusarium oxysporum* from nosocomial infections (O'Donnell et al. 2004) each demonstrated substantially clonal populations. Thus, nosocomial outbreaks appear to be the result of clonal propagation of an opportunistic pathogen as a result of a genetic bottleneck that allows one of a few variants to disseminate through a population of susceptible hospital patients as hosts. Our results confirm that passage through a susceptible host is capable of producing a genetic bottleneck in a population of an opportunistic pathogen.

The serial propagation of *A. flavus* through wax moth larvae is intended as a model system to study the repeated transmission of an opportunistic pathogen through host to which it is pathogenic but not well adapted. Such serial passage experiments have previously been interpreted as useful for the study of pathogen adaptation and emerging diseases (Ebert 1998; Scully & Bidochka 2005). Theoretically, novel diseases emerge when virulent, host-specific facultative and obligate pathogens evolve from less virulent, less-specialized opportunistic pathogens (Scheffer 1991). Our research suggests that repeated infection of a coincidental host may act as a genetic bottleneck on a population of an opportunistic pathogen, creating the opportunity for the allopatric evolution of virulent new strains and eventually novel disease-causing microorganisms.

Chapter IV:

A cysteine/methionine auxotroph of the opportunistic fungus *Aspergillus flavus* is associated with host-range restriction: a model for emerging diseases

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Abstract

The evolution of host specialization in pathogens is a topic of considerable interest, particularly since it can represent a decisive step in the emergence of infectious diseases. *Aspergillus flavus* is an opportunistic fungus capable of infecting a wide variety of hosts including plants, insects, and mammals, although with low virulence. Here the derivation of an *A. flavus* strain that exhibits severe host restriction is reported. This strain exhibited a severe diminution or a complete lack of conidial production on a variety of standard agar media and on various plant species. However, it retained its ability to infect insects from various orders and to re-emerge from and adequately conidiate on the insect cadavers as a culmination of the pathogenic life cycle. This strain, demonstrating insect-dependent conidiation, was discovered to be a cysteine/methionine auxotroph due to an inability to reduce sulfate to sulfite. However, other *A. flavus* auxotrophs tested for plant and insect host range failed to show insect-dependent conidiation. An association between this specific auxotroph and a decreased host range is shown, emphasizing the role of nutrition in the host-pathogen relationship with respect to host restriction and evolution toward obligate pathogenesis.

Introduction

In terms of host-pathogen relationships, microorganisms can be classified into one of three categories: opportunistic, facultative, or obligate pathogens (Scheffer 1991). Opportunistic pathogens persist on a wide range of organic substrates but exhibit low virulence toward a broad array of living hosts, particularly if the host is injured or immunocompromised. Facultative pathogens efficiently colonize and produce disease in a narrow range of healthy hosts, although they are capable of surviving outside the host. Microorganisms that require a living host for survival and replication constitute the obligate pathogens, and generally have narrow host ranges. Specialized pathogens, such as the obligate and facultative pathogens, probably evolved from the less specialized opportunistic pathogens, representing a theoretical framework for the emergence of infectious diseases and host specialization (Scheffer 1991).

To study the evolution of host specificity and the development of infectious diseases, we utilized an insect-fungal model of infection involving the fungus *Aspergillus flavus* as the pathogen and wax moth larvae (*Galleria mellonella* (Lepidoptera)), as the insect host. *A. flavus* is an opportunistic fungal pathogen capable of thriving saprobially as well as infecting a wide variety of living hosts, including plants, insects, and mammals, albeit with low virulence. *A. flavus* is an ideal model pathogen for the study of adaptation and host restriction, since it possesses a broad spectrum of protein- and polysaccharide-hydrolyzing enzymes for exploiting available living and non-living organic resources (St. Leger et al. 2000). With respect to insect pathogenicity, *A. flavus* is an opportunistic pathogen of silkworms, grasshoppers, houseflies, and mealy bugs among others (Gupta & Gopal 2002). *A. flavus* is the causative agent of aspergillosis documented in the silkworm *Bombyx mori* (Kumar et al. 2004). *A. flavus* infection of insects involves conidium germination on the insect surface, followed by a breach of the cuticle and access to the nutrient-rich hemocoel. Inside the infected insect, the hyphae multiply extensively, causing death and subsequent mycelial emergence from and mummification of the cadaver. The emergent mycelia conidiate on the surface of the cadaver, providing the transmissible propagules

dispersed to other hosts for the continuation of the pathogenic cycle (Kumar et al. 2004). In the event that a suitable host is not available, the conidia germinate and grow saprobially.

In this study, we employed a serial propagation scheme involving the continuous passage of *A. flavus* through larvae of *G. mellonella*, mimicking the repeated infection and transmission of a disease-causing, opportunistic pathogen through a single species of a susceptible host. The result was a strain of *A. flavus* that exhibited a severe diminution in conidial production on standard artificial media, while retaining pathogenicity toward the insect host, including conidial production on the dead host during the final stage of pathogenesis. In addition, it demonstrated a reduction in host range as a plant pathogen compared to the original strain as well as an auxotrophic requirement for cysteine/methionine due to an inability to reduce sulfate to sulfite. In essence, this strain represents the progression by an opportunistic fungus towards obligate insect pathogenesis in that it can only efficiently complete its life cycle (i.e. mycelial growth followed by conidial production) as a consequence of infection of an insect host. In the opportunistic pathogen *A. flavus*, we show an association between auxotrophy and severe host restriction constituting a shift toward obligate pathogenesis that emphasizes the role of nutrition in evolving host-pathogen relationships.

Materials and Methods

Fungi, insect, and plant species

This study utilized *Aspergillus flavus* strains 6982, 4336, 9308 (University of Alberta Microfungus Collection and Herbarium), 1010, 2159 (Collection of Entomopathogenic Fungal Cultures USDA-ARS Plant Protection Research Unit), 46109 (*his*-), 46110 (*ile-val*-), 46111 (*pro*-), 46112 (*phe*-), and 46114 (*met*-) (American Type Culture Collection) (Papa 1980), *Aspergillus fumigatus* FR 2923-97 (Rosehart et al., 2002), and *Emericella nidulans* 7677 (University of Alberta Microfungus Collection and Herbarium). All strains were maintained on Petri dishes containing 10ml volumes of Potato Dextrose Agar (PDA) incubated at 30°C for 7 d.

The following insect and plant species were used in this study: mealworms (*Tenebrio molitor* (Coleoptera)), blowfly larvae (*Sarcophaga bullata* (Diptera)), and crickets (*Acheta domestica* (Orthoptera)) from Ward's Scientific, wax moth larvae (*Galleria mellonella* (Lepidoptera)) from Peterborough Live Bait (Peterborough, Ontario), and milkweed bugs (*Oncopeltus fasciatus* (Hemiptera)) from Boreal, tomato (*Lycopersicon sp.*), corn (*Zea sp.*), green bean (*Phaseolus sp.*), pea (*Pisum sp.*), and cucumber (*Cucumis sp.*) from Zehrs (St. Catharines, Ontario), and alfalfa (*Medicago sp.* (Boreal)).

Serial propagation of *A. flavus* through *G. mellonella* larvae

To propagate *A. flavus* 6982 through *G. mellonella*, 25 larvae were topically inoculated on the dorsal surface with 5 μ l of 1×10^5 conidia ml^{-1} suspension, placed separately into clean plastic vials, and incubated at 30°C. At death, insects were washed for 1 minute in 1% (v/v) NaOCl followed by 2 washings of 2 min each in sterile dH₂O. (This procedure effectively removed surface conidia from insect cadavers, allowing only cuticle-breeching, pathogenic conidia to survive, since control experiments showed that *A. flavus* failed to grow on previously frozen, dead larvae inoculated and surface sterilized in this manner.) Emergent conidia from the first insect to die were harvested in 3ml of a 0.01% (v/v) Triton X-100--0.02% (w/v) chloramphenicol solution. After quantifying the suspension microscopically with a hemacytometer, 25 larvae were topically inoculated to obtain the next generation of fungal passage through the insect host. Five replicate lineages of this scheme were performed, each consisting of six generations of fungal passage through the host. Fungal samples from each generation of each lineage were stored at -80°C in 25% glycerol and subcultured onto PDA. The initial conidial suspension used in this scheme was mutagenized by exposure to UV-A light (400 $\mu\text{W}/\text{cm}^2$) for 3 min, resulting in 80% mortality of conidia (see Appendix A).

From this artificial selection scheme, a single strain of *A. flavus* was derived that exhibited a drastic reduction in conidial production on PDA, while producing conidia on the infected insect cadaver as the culmination of the pathogenic cycle. This strain was designated

Af6982con^{ins} of *A. flavus* because it demonstrated efficient conidial production on the infected insect cadaver but not on PDA. *Af6982con^{ins}* demonstrated a similar colony phenotype when subcultured onto nutrient agar (NA; Difco) and yeast peptone dextrose agar (YPD; 0.2% yeast extract, 1% peptone, 2% dextrose, 1.5% agar) and demonstrated no growth on Czapek solution agar (Cz; Difco).

DNA extraction, ITS amplification, and Amplified-fragment length polymorphism (AFLP) analysis

To confirm that the derived strain was not a contaminant, the DNA was extracted using the DNeasy tissue extraction kit (Qiagen), and the ITS region was amplified and sequenced using standard protocols and ITS primers (White et al. 1990).

To further confirm the relationship of the derived strain to the parental strain as well as to other strains and species of *Aspergillus*, we used AFLP analysis. Genomic DNA of *A. flavus*, *A. fumigatus*, and *E. nidulans* isolates was prepared using the DNeasy tissue extraction kit (Qiagen). Five hundred nanograms of fungal DNA were digested with 1 U EcoRI and 1 U MseI in a total volume of 10µl following the manufacturer's instructions (New England Biolabs). Following digestion, reactions were incubated at 70°C for 15 min. Ligation was performed in a total volume of 20µl by adding 2pmol of E adapter, 20pmol of M adapter (Table 3-1), and 0.4U of T4 DNA ligase (New England Biolabs) to the total volume of the digestion reaction. The ligation reactions were incubated at 20°C for 2 h. The digestion-ligation products were diluted 1:10 with TE (10mM Tris-HCl, 1mM EDTA, pH 8.0).

Pre-amplification was carried out in a total volume of 30µl, containing 5µl of diluted digestion-ligation product, 0.5µM primer EO, 0.5µM primer MO (Table 3-1), and 15µl of Taq PCR Master Mix (Qiagen). The PCR conditions were: 20 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s. The preamplification products were diluted 1:10 with TE.

Eleven selective amplification reactions were performed using the following primer combinations: EO/M+CAG, E+A/M+A, E+A/M+CAG, E+GC/MO, E+GC/M+T, E+GC/M+A,

E+GC/M+CAG, E+AG/MO, E+AG/M+T, E+AG/M+A, E+AG/M+CAG (Table 3-1). The selective amplification reactions contained 5µl diluted preamplification product, 0.5µM of each primer, and 11µl Taq PCR Master Mix (Qiagen) in a total volume of 22µl. The PCR conditions were: 6 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s; 6 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s; 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s; followed by a final extension at 72°C for 5 min.

The selective amplification products were analyzed on 10% (19:1) denaturing polyacrylamide gels (Sequi-Gen GT Nucleic Acid Electrophoresis Cell, BioRad), which were subsequently silver-stained (Blum et al. 1987).

A similarity matrix using simple matching coefficients was constructed using NYSTS 2.1 after scoring the bands “1” for presence and “0” for absence. Cluster analysis was performed using unweighted pair-group method using arithmetic averages (UPGMA).

Insect and plant bioassay

Virulence toward *G. mellonella* larvae was determined by topical application of 25 larvae with 5µl of 1×10^8 conidia ml⁻¹ and recording percentage mortality at day 10. Fisher’s exact tests or χ^2 tests were used to compare the percentage mortality of *A. flavus* strains to each other and to the control bioassay, which was topical application of 5µl of 0.01% (v/v) Triton X-100.

Pathogenicity tests against all insect species were performed by injecting conidial suspensions (3µl of 1×10^6 conidia ml⁻¹) into the hemocoel using a clean syringe (gauge=22). All insects were placed individually in clean plastic vials, incubated at 30°C, and monitored daily for mortality and subsequent conidiation on the cadaver. Inoculation via injection was utilized to ensure infection.

For plant pathogenicity tests, various plant species were washed in 1% NaOCl for 1 minute followed by three 1 min washings in sterile dH₂O. Plants were inoculated by piercing the tissue with a sterilized needle, followed by inoculation of the wounded area with 2µl of a 1×10^6 conidia ml⁻¹ suspension. Some suspensions were supplemented with a 10mM final concentration

of either cysteine or methionine. The plants were incubated on 1% water agar at 30°C for 7 d before assessing mycelial growth and conidiation.

For all plant and insect bioassays utilizing auxotrophic *A. flavus* strains, emergent conidia were tested to determine if they retained the auxotrophic phenotype. The conidia were subcultured onto Cz and Cz supplemented with 10mM of the required amino acid. Lack of growth in the absence of supplementation confirmed the auxotrophic phenotype. Any plants or insects with conidia that failed to display an auxotrophic phenotype were omitted from the data since this indicated reversion.

Conidial counts

To quantify conidial production of a subculture grown on PDA with or without 10mM cysteine or methionine, a 2.54 cm diameter agar plug was removed from halfway between the center of the Petri dish and the perimeter. The plug was homogenized in 5ml 0.01 % Triton X-100 for 1 min in a 50ml polystyrene tube with a motorized homogenizer (Greiner Scientific). Conidia were counted using a hemacytometer.

Quantification of conidia produced on *G. mellonella* and *Zea sp.* was performed by vortexing a conidiated insect cadaver or corn kernel in 3ml of 0.01% Triton X-100--0.02% chloramphenicol. The resulting suspension was counted using a hemacytometer.

Microscopy

Samples of fungal material from *G. mellonella* larvae and PDA plates were stained with lactophenol cotton blue and examined under a bright-field microscope.

Detection of auxotroph phenotype and reversion

Wells of 96-well plates were filled with 200µl of Cz and individually supplemented with 1 µmol of one of the following amino acids: L-α alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. Wells were inoculated with 5µl of a 1×10^6 conidia ml⁻¹ suspension.

Screening for auxotrophic reversion was performed by plating 50 μ l of 1x10⁶ conidia ml⁻¹ *Af6982con^{ins}* suspension onto each of 40 Cz Petri dishes and observing fungal growth.

Sulfur assimilation enzyme assays

Flasks containing 100ml of YPD broth (0.2% yeast extract, 1% peptone, 2% dextrose) were inoculated with 50 μ l of 1x10⁶ conidia ml⁻¹ and incubated at 27°C shaking at 250rpm for 3 d. Fungi were then filtered from the broth, washed with Czapek-Dox broth (Difco), and transferred to flasks containing 100ml of Czapek-Dox broth incubated for 2 days at 27°C and 250rpm. After filtering from the media, the fungi were crushed in liquid nitrogen and the resulting material was used to assay the activity of ATP sulfurylase as described by Segel et al. (1987), the conversion of sulfate to sulfite (Breton & Surdin-Kerjan 1977), and sulfite reductase (De Vito & Dreyfuss 1964). Sulfate permease function was assessed based on the inhibition of growth by the toxic sulfate analogue chromate on Cz agar containing 10mM methionine and 10mM potassium chromate as described by Arst (1968).

Results

A strain of *A. flavus* demonstrating insect-dependent conidiation

A. flavus was subjected to serial propagation through *G. mellonella* larvae for six generations. During serial passage, one of the lineages produced a strain that demonstrated a remarkable characteristic. Although this strain remained pathogenic toward the larvae, producing conidia on the surface of the insect cadaver as the final stage of pathogenesis, it demonstrated a drastic reduction in conidial production when grown on PDA (Figure 4-1). A similar reduction was observed on a variety of other agar media, namely YPD and NA (see Appendix D), and unlike the original strain, no growth occurred on Cz. Conidial production exhibited by this strain when grown on PDA was 75-fold less than that of the original strain previous to passage through the insect host (Figure 4-2). Conversely, the amount of conidia produced by this strain on the insect cadaver at the completion of the pathogenic cycle was much higher, exhibiting only a 10-fold reduction compared to the parental strain by either topical application or injection (Figure 4-

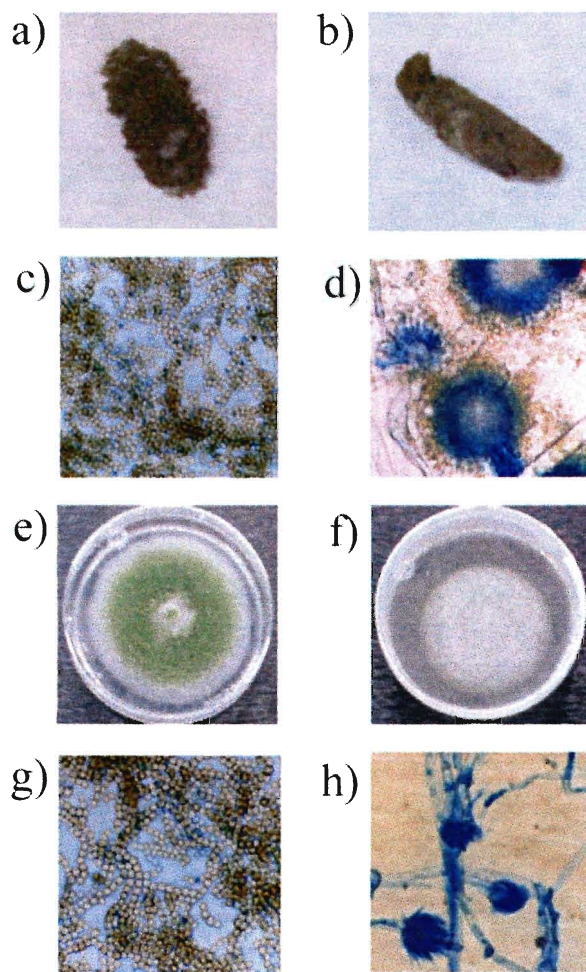


Figure 4-1: Conidiation of *Af6982* and *Af6982con^{ins}* on *G. mellonella* larvae (injected with $3\mu\text{l}$ 1×10^6 conidia ml^{-1}) or on PDA. Top panel. Mycelial emergence and conidiation on larvae by (a) *Af6982* and (b) *Af6982con^{ins}*. Second panel. Microscopic examination of (c) *Af6982* and (d) *Af6982con^{ins}* from infected larvae. Third panel. Mycelial growth and conidiation on PDA by (e) *Af6982* and (f) *Af6982con^{ins}*. Fourth panel. Microscopic examination of (g) *Af6982* and (h) *Af6982con^{ins}* from PDA.

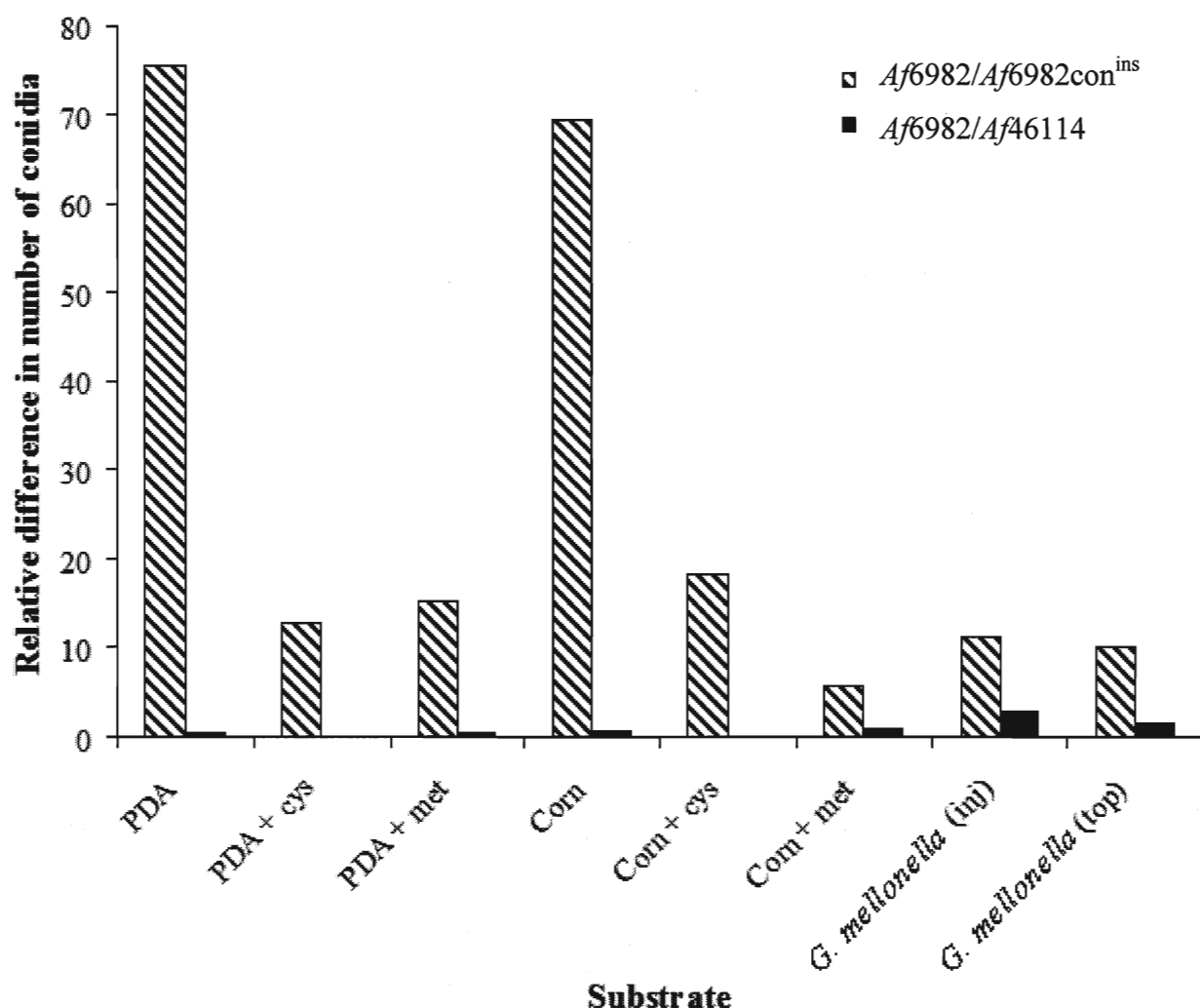


Figure 4-2: Relative difference in number of conidia produced by *Af6982* and *Af6982con^{ins}* or *Af46114* (*met*-) on PDA, corn, and *G. mellonella* larvae. The relative difference was calculated as the number of conidia produced by *Af6982* divided by the number of conidia produced by *Af6982con^{ins}* or *Af46114*. PDA and corn were supplemented with 10mM of either cysteine (cys) or methionine (met). The amount of conidia on *G. mellonella* larvae was assessed after both topical application (5 μ l of 1×10^8 conidia ml⁻¹) and injection (3 μ l of 1×10^6 conidia ml⁻¹). For all substrates, the amount of conidia was calculated as an average of three replicates.

2). These characteristics were displayed by this strain at each of generations 2-6 of passage through the insect (see Appendix D).

The BLAST search results of the ITS sequence of the selected strain exhibited a 97% similarity with the ITS sequences of other *A. flavus* strains (GenBank accession no. AY521473; see Appendix E). Because of its dependency on the insect host for adequate conidial production, this strain is designated *Af6982con*^{ins}.

Virulence and host range of *Af6982con*^{ins}

The virulence of *Af6982con*^{ins} towards *G. mellonella* larvae (64% mortality at day 10) remained comparable to that of *Af6982* (48% mortality at day 10) ($p = 0.39$, Fisher's exact test) and statistically significant compared to a control inoculation ($p = 0.001$ and $p = 0.03$, respectively, Fisher's exact test) during topical application bioassays (5 μ l of 1×10^8 conidia ml⁻¹).

Insects of various orders as well as a range of plant species were inoculated with *Af6982* and *Af6982con*^{ins} in order to determine host ranges established by the essential pathogenic property of conidiation on the infected host indicative of successful colonization. The results indicated that *Af6982con*^{ins} and its parental strain *Af6982* both possessed the ability to conidiate on a variety of infected insects (injected with 3 μ l of 1×10^6 conidia ml⁻¹). However, unlike strain *Af6982*, *Af6982con*^{ins} failed to produce lesions or exhibit any growth on the infected plant species except for the corn kernels on which it produced some mycelia and exceptionally few conidia (Table 4-1; Figure 4-2).

Amino acid supplementation of *Af6982con*^{ins}

Af6982con^{ins} was grown on Cz supplemented with single amino acids in order to detect an auxotrophic phenotype. Whereas the parental strain exhibited growth regardless of amino acid supplementation, *Af6982con*^{ins} showed no growth on Cz except when supplemented with either cysteine or methionine (Figure 4-3).

Table 4-1: Conidiation of *A. flavus* strains *Af6982*, *Af6982con^{ins}*, the revertant, and *Af46114* (*met-*) on insects of various orders (injected with 3µl of 1x10⁶ conidia ml⁻¹) and a range of plant species. Data are representative of at least two independent trials. (+++ much conidiation; ++ moderate conidiation; + little conidiation; - no conidiation)

Kingdom	Order	Common name	Conidiation*			
			<i>Af6982</i>	<i>Af6982con^{ins}</i>	Revertant	<i>Af46114</i>
Animalia	Coleoptera	Mealworm	+++	++	+++	+++
Animalia	Diptera	Blowfly larvae	++	+	++	++
Animalia	Lepidoptera	Wax moth larvae	+++	++	+++	+++
Animalia	Orthoptera	Cricket	-	-	-	-
Plantae	Solanales	Tomato	+++	-	+++	+
Plantae	Poales	Corn	+++	+	+++	+++
Plantae	Fabales	Alfalfa	++	-	++	++
Plantae	Fabales	Pea	++	-	++	+
Plantae	Fabales	Green bean	++	-	++	-
Plantae	Cucurbitales	Cucumber	+	-	+	-

*Note: Here we indicate that conidiation is the culmination of mycelial growth.

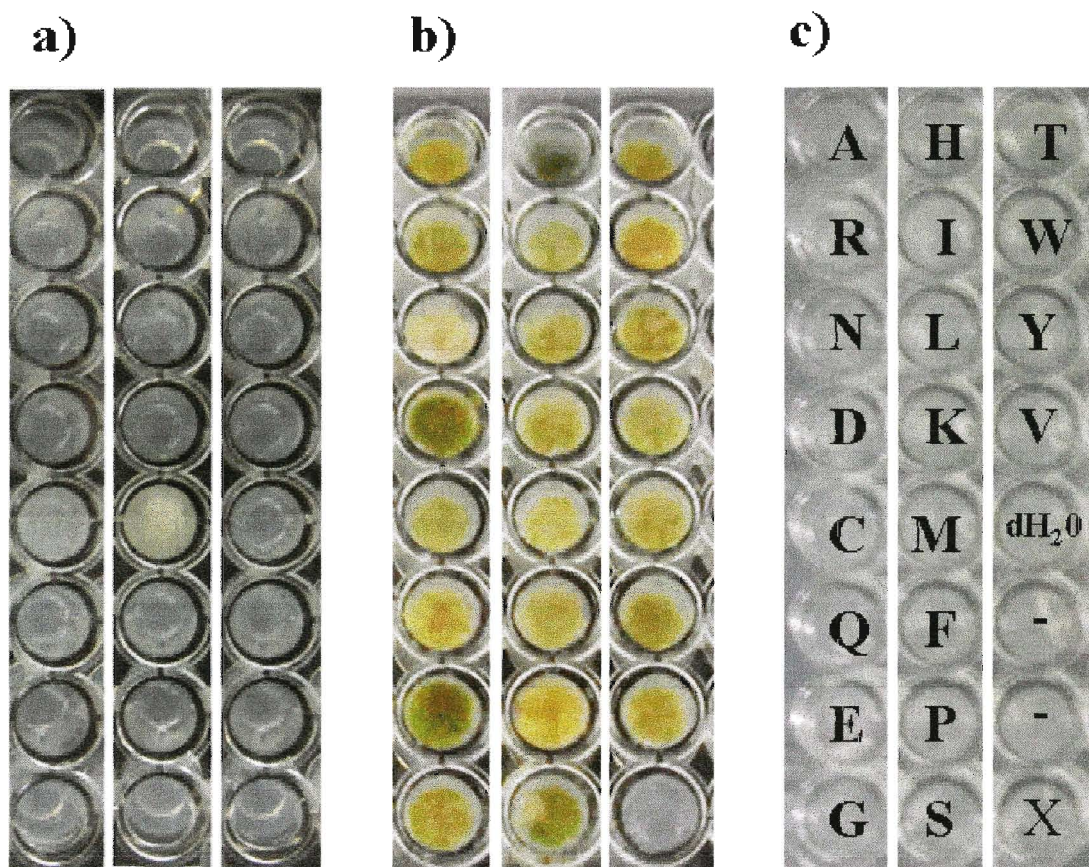


Figure 4-3: Growth of (a) *Af6982con^{ins}* and (b) *Af6982* on Cz media supplemented with various amino acids. Wells containing 200 μ l of Cz agar were supplemented with 10mM of the amino acid indicated in the key (c). Wells designated “-” were not supplemented. All wells except “X” were inoculated with 5 μ l of a 1×10^6 conidia ml^{-1} and incubated at 30°C for 3 d.

Although supplementation of *Af6982con*^{ins} with either cysteine or methionine failed to restore pathogenicity toward most plant hosts, on PDA and corn, conidial production relative to *Af6982* was brought to a level similar to that generated on the insect host (Figure 4-2).

Sulfur assimilation enzyme activity of *Af6982con*^{ins}

The ability of *Af6982con*^{ins} to import sulfate and convert it to cysteine or methionine was assessed by analyzing the functionality of the enzymes of the sulfur assimilation pathway (Figure 4-4a). Both *Af6982* and *Af6982con*^{ins} failed to grow in the presence of chromate, a toxic sulfate analogue, indicating a functional sulfate permease (Figure 4-4b). While both *Af6982* and *Af6982con*^{ins} demonstrated comparable levels of ATP sulfurylase (Figure 4-4c, $t = 0.74$, $p = 0.26$) and sulfite reductase (Figure 4-4e, $t = 0.13$, $p = 0.46$) activity, *Af6982con*^{ins} failed to show any sulfate to sulfite reduction activity (Figure 4-4d).

Spontaneous reversion

Screening for spontaneous reversion of *Af6982con*^{ins} by plating conidia on Cz produced one revertant in 2×10^6 conidia that exhibited full recovery of the growth and conidiation properties of the parent strain when grown on PDA, Cz, NA, and YPD. The revertant also demonstrated full recovery of the conidiation and pathogenicity properties of the parental strain *Af6982* when assayed against various plant species, while maintaining pathogenicity toward insects (Table 4-1). In addition, the revertant retained a level of virulence towards *G. mellonella* larvae that was statistically different from a control inoculation (32% mortality by day 10, $p = 0.02$, Fisher's exact test) and statistically equivalent to that of the original strain *Af6982* and *Af6982con*^{ins} ($p = 0.08$, χ^2 test).

AFLP analysis

Cluster analysis based on the presence or absence of AFLP bands (Figure 4-5) indicated that isolates of the original strain *Af6982*, the strain demonstrating insect- dependent conidiation *Af6982con*^{ins}, and the revertant possessed a 99% similarity by simple matching coefficients. The similarity of these isolates to other *A. flavus* strains ranged from 57% to 94%, while their

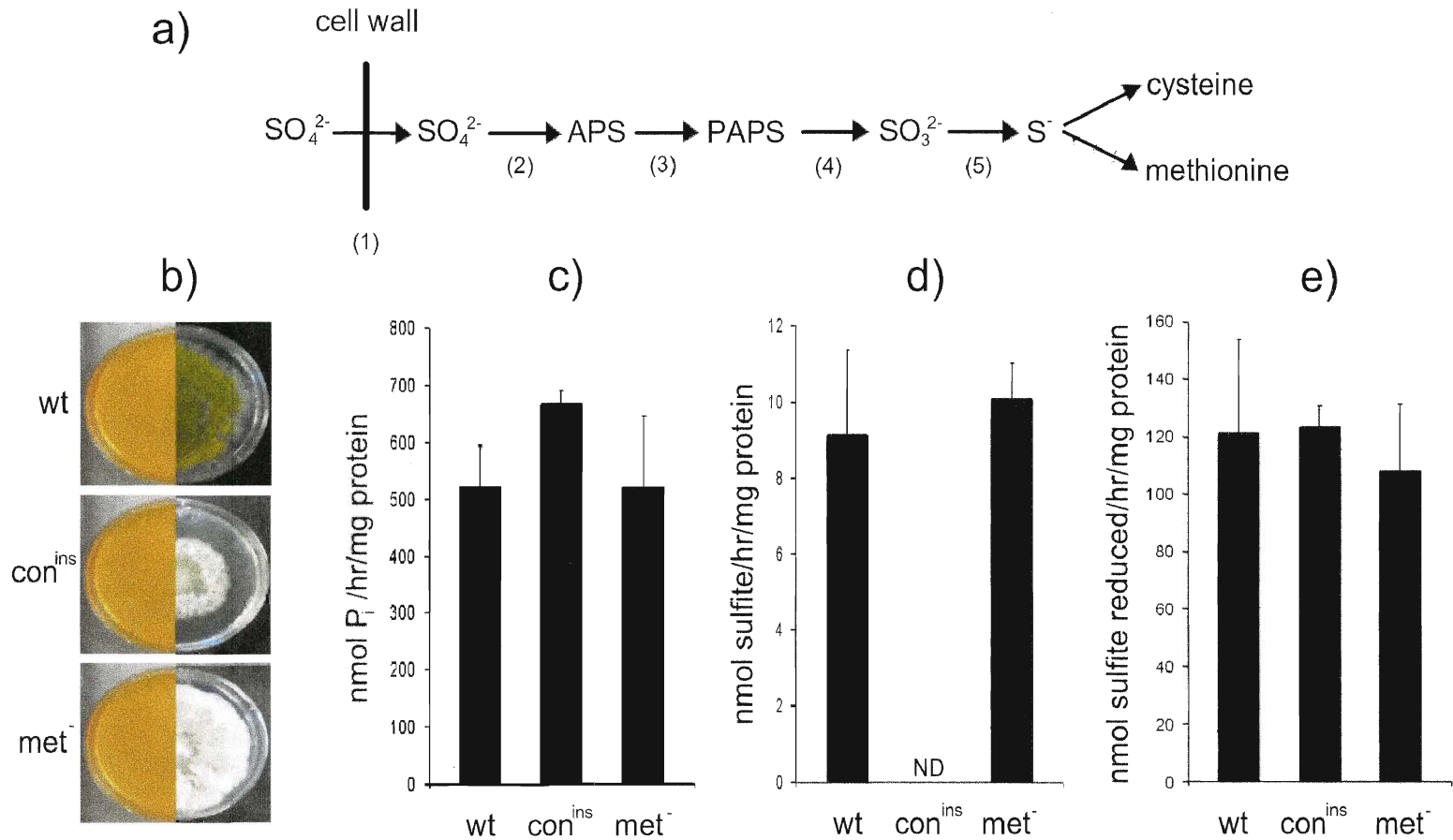


Figure 4-4: Functionality of sulfur assimilation pathway enzymes in *Af6982* (wt), *Af6982con^{ins}* (con^{ins}), and *Af46114* (met^-). (a) Sulfur assimilation pathway of *Aspergillus nidulans*; (1) sulfate permeases, (2) ATP sulfurylase, (3) APS kinase, (4) PAPS reductase, (5) sulfite reductase. APS = adenosine 5' phosphosulfate, PAPS = 3'-phosphoadenosine-5'-phosphosulfate (modified from Marzluf, 1997). (b) Growth in the presence (left) and absence (right) of $\text{K}_2\text{Cr}_2\text{O}_7$. (c) ATP sulfurylase activity. (d) Sulfate to sulfite reduction activity. (e) Sulfite reductase activity. ND represents no enzyme activity detected. Values are an average of three independent trials.

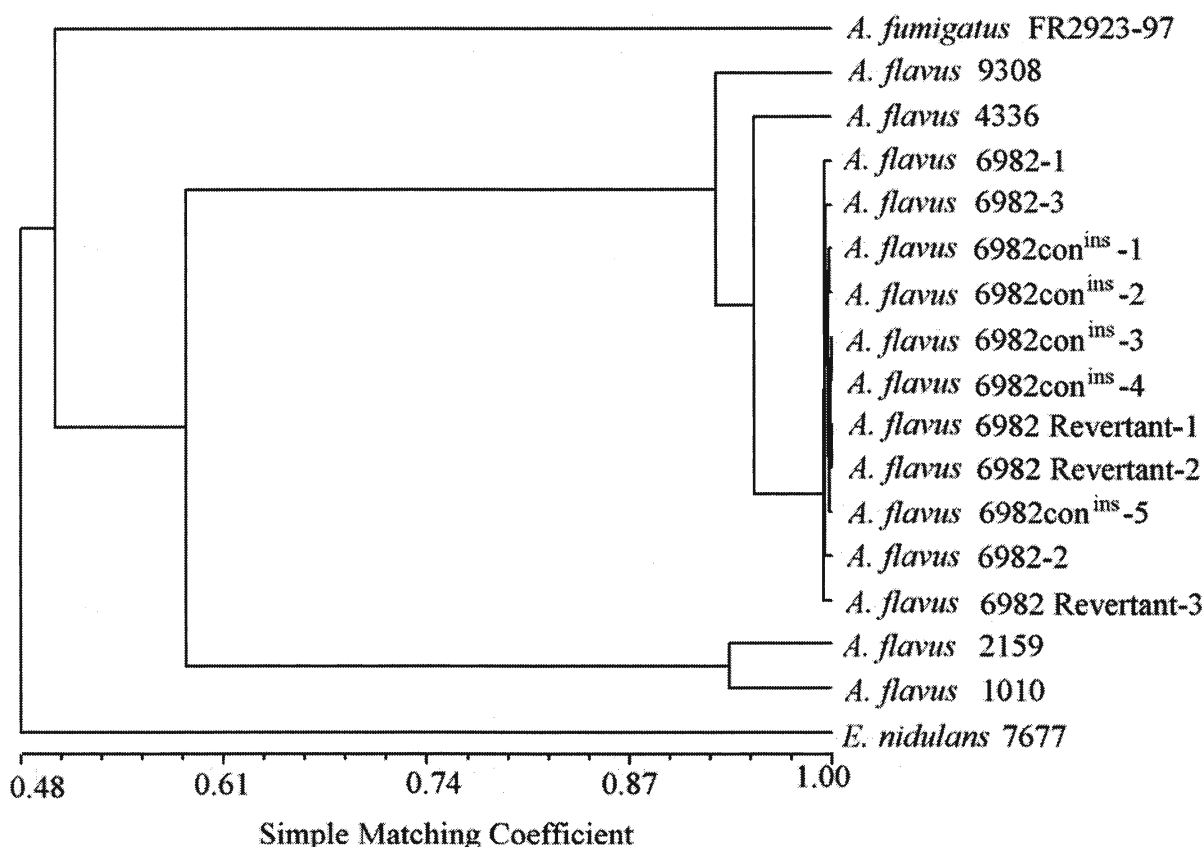


Figure 4-5: UPGMA cluster analysis of *Aspergillus fumigatus*, *Emericella nidulans*, and single or multiple isolates of *A. flavus* based on simple matching coefficients generated from the presence or absence of AFLP bands. The number after the strain number refers to one of the single colony isolates of *Af*6982, *Af*6982con^{ins}, or the revertant.

similarity to *A. fumigatus* and *E. nidulans* was 49% and 47%, respectively. Although differences were observed between the isolates of *Af6982*, *Af6982con^{ins}*, and the revertant, there were no diagnostic polymorphisms that distinguished the isolates of the original strain from those of the strain demonstrating insect-dependent conidiation.

Virulence and host range of *A. flavus* auxotrophs

Five *A. flavus* auxotrophs with requirements for histidine, proline, isoleucine and valine, phenylalanine, or methionine (Papa, 1980; and confirmed in this study, see Appendix F) all exhibited minimal to no growth on Cz. All five auxotrophs exhibited infection and substantial conidiation on *G. mellonella* cadavers when injected or applied topically to the cuticle. Three of the five auxotrophic strains demonstrated a statistically significant percent mortality (Table 4-2) compared a control inoculation during topical application bioassays (5µl of 1×10^8 conidia ml⁻¹). However, unlike *Af6982con^{ins}*, most of these auxotrophs retained the ability to infect alfalfa (Table 4-2). Further investigation of *A. flavus* 46114, which demonstrated an auxotrophic requirement for methionine similar to that of *Af6982con^{ins}*, showed an ability to infect a variety of both plants and insects (Table 4-1), and exhibited no diminution in conidial production on PDA, corn, or *G. mellonella* larvae relative to *Af6982* (Figure 4-2). As with *Af6982*, *Af46114* failed to grow in the presence of chromate (Figure 4-4b), demonstrating a functional sulfate permease, and exhibited comparable levels of ATP sulfurlyase (Figure 4-4c, $t = 0.01$, $p = 0.50$), sulfate to sulfite reduction (Figure 4-4d, $t = 0.66$, $p = 0.28$), and sulfite reductase activity (Figure 4-4e, $t = 0.56$, $p = 0.30$).

Discussion

This study reports the derivation of a strain of the opportunistic fungus *A. flavus* that exhibited characteristics similar to those of obligate pathogens. The properties displayed by this strain, designated *Af6982con^{ins}*, include a drastic reduction in its ability to produce conidia on artificial media paralleled by adequate conidiation as the end result of the pathogenic cycle when restricted to the insect host. In addition to its decreased saprobic abilities, *Af6982con^{ins}* also

Table 4-2: Conidiation properties and virulence of *A. flavus* auxotrophs with respect to alfalfa (*Medicago sp.*) and *G. mellonella* hosts. Virulence was determined as percent mortality by day 10 after topical application of 5µl of 1×10^8 conidia ml⁻¹. (+ conidiation; - no conidiation).

Strain	Phenotype	Conidiation on Alfalfa	Conidiation on <i>G. mellonella</i>		Virulence toward <i>G. mellonella</i>	
			Injection	Top. appl.	% mortality	P-value
<i>Af6982</i>	wild type	+	+	+	48%*	0.03*
<i>Af6982con^{ins}</i>	<i>cys/met-</i>	-	+	+	64%*	0.001*
Revertant	wild type	+	+	+	32%*	0.02*
<i>Af46109</i>	<i>his-</i>	-	+	+	24%	0.72
<i>Af46110</i>	<i>ile- val-</i>	+	+	+	12%	0.99
<i>Af46111</i>	<i>pro-</i>	+	+	+	56%*	0.007*
<i>Af46112</i>	<i>phe-</i>	+	+	+	60%*	0.003*
<i>Af46114</i>	<i>met-</i>	+	+	+	53%*	0.012*

* represents statistically significant percent mortality compared to control inoculation (topical application of 5µl of 0.01% Triton X-100) using Fisher's exact test.

demonstrated a decreased host range compared to the original strain *Af6982*. Whereas *Af6982* infected, produced mycelia, and generated conidia on insects from different orders and various plant species, *Af6982con^{ins}* generated conidia most efficiently on insects, while failing to produce mycelia and conidia on most plant species. Furthermore, *Af6982con^{ins}* demonstrated these properties while maintaining virulence toward *G. mellonella* larvae at a level comparable to that of the original strain *Af6982*. Thus, while retaining pathogenicity toward insects, *Af6982con^{ins}* demonstrated a dependency on the insect host as its most robust option for reproduction as the culmination of its pathogenic cycle. Unlike *Af6982*, neither growth on standard artificial media nor reproduction as a plant pathogen represented a suitable option. These characteristics of *Af6982con^{ins}* constitute a shift towards obligate-insect pathogenesis.

Supplementation of Cz with various amino acids revealed that *Af6982con^{ins}* was a cysteine/methionine auxotroph. This nutrient deficiency provides a plausible explanation for its dependency on the insect host for reproduction. Such nutrient requirements suggest that infection and propagation through the larval host allowed this auxotroph to grow and conidiate as it gained the required amino acids from the host, most likely from the protein- and amino acid-rich insect cuticle or hemolymph (Hackman & Goldberg 1976; Hanzal & Jegorov 1991; Paterson et al. 1994). However, neither standard artificial media nor plant species were able to provide *Af6982con^{ins}* with the nutrients required for adequate conidiation. Accordingly, *Af6982con^{ins}* exhibited properties similar to those of obligate pathogens because it was severely impeded in its ability to complete its lifecycle via the production of conidia unless it was allowed to infect, colonize, gain nutrients from, and finally re-emerge from the insect to conidiate on the cadaver surface.

Many pathogens designated as obligate can proliferate on artificial media when they are provided with the appropriate nutrients. For example, the obligate mammalian pathogens *Mycobacterium tuberculosis* and *Yersinia pestis* can be cultured on Lowenstein-Jensen medium and TMH medium respectively (Qui et al. 2005; Tanoue et al. 2002). *Cronartium quercuum*, the

obligate fungal pathogen of oak tree leaves, can be cultivated on PGY medium (Warren & Covert 2004). In principle, the cysteine/methionine auxotroph described in this study behaves no differently. Besides demonstrating a restricted host range, *Af6982con^{ins}* requires enhanced artificial media for culture growth. These characteristics show similarity with other obligate pathogens and demonstrate an evolution toward obligate pathogenesis.

Unlike *Af6982con^{ins}*, a variety of other *A. flavus* auxotrophs demonstrating nutrient deficiencies did not show a diminished host range. Four of the five auxotrophs demonstrated mycelial growth and conidiation on the insect host *G. mellonella* larvae as well as the ability to infect and conidiate on alfalfa leaves. Specifically, *A. flavus* strain 46114, which displayed an auxotrophic requirement for methionine similar to that of *Af6982con^{ins}*, demonstrated that its nutrient deficiency neither reduced the host range of *Af46114* nor hindered its ability to produce conidia on a variety of substrates. Enzyme assays revealed that unlike *Af46114*, *Af6982con^{ins}* is deficient in the ability to reduce sulfate to sulfite, indicating that the metabolic cause of auxotrophy in these two strains is different. Such metabolic differences are a likely explanation for the observed differences in host range.

Of the twenty essential amino acids, cysteine and methionine are the only two that contain sulfur. Not surprisingly, their biosynthetic and metabolic pathways are highly interconnected (Ono et al. 1999), leading to the hypothesis that the cysteine/methionine auxotrophy of *Af6982con^{ins}* was a result of a single-gene mutation in a common biosynthetic pathway. We obtained a spontaneous revertant of *Af6982con^{ins}* that regained the characteristics of the original opportunistic pathogen *Af6982*, namely conidiation on artificial media and plant species while preserving insect pathogenicity and a similar level of virulence toward *G. mellonella* larvae. The recovery of a spontaneous revertant implied that the obligate pathogen characteristics of *Af6982con^{ins}* were due to a single gene mutation, since the probability of obtaining a spontaneous revertant after multiple mutations was unlikely.

Because fungi are able to interconvert cysteine and methionine (Ono et al. 1999), a single-gene mutation resulting in auxotrophy that is recovered by either cysteine or methionine implies a mutation in the sulfur assimilation pathway essential for the synthesis of either amino acid (Marzluf 1997). Indeed, we showed that *Af6982con^{ins}* was unable to reduce sulfate to sulfite. We hypothesize that the auxotrophy of *Af6982con^{ins}* is due to an incapacitating mutation in one of the enzymes essential for the reduction of sulfate to sulfite, presumably either adenosine 5'-phosphosulfate (APS) kinase or 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, since ATP sulfurlyase activity is detected in *Af6982con^{ins}*. Further experiments are underway to genetically characterize this mutation.

The high level of similarity between the original opportunistic strain *Af6982*, the strain demonstrating insect-dependent conidiation *Af6982con^{ins}*, and the revertant indicated by the AFLP cluster analysis suggested that they are derived from the same strain (Janssen et al. 1997; Rademaker et al. 2000; Savelkoul et al. 1999). In all likelihood, a mutant produced during the UV mutagenesis of the initial conidial suspension was selected during passage through the insect that resulted in the derivation of a strain, which displayed insect-dependent conidiation. Subsequent reversion of the mutation allowed for recovery of the revertant. Although a small number of differences were observed among the isolates of the *Af6982*, *Af6982con^{ins}*, and the revertant, this degree of dissimilarity has been observed between multiple isolates of a single strain of other asexually reproducing microbes (Janssen et al. 1997; Rademaker et al. 2000; Savelkoul et al. 1999). These differences may be due to noise or hysteresis in this PCR-based technique. Alternatively, they may be legitimately due to mutations as asexually reproducing microbes are not genetically static during subculturing.

Our results indicated an association between a single gene mutation resulting in auxotrophy and host restriction, resulting in evolution towards obligate-insect pathogenesis by an opportunistic pathogen. As shown in Figure 4-6, such a mutation may represent a decisive step in the emergence of a highly virulent obligate pathogen as it may provide selection pressure for

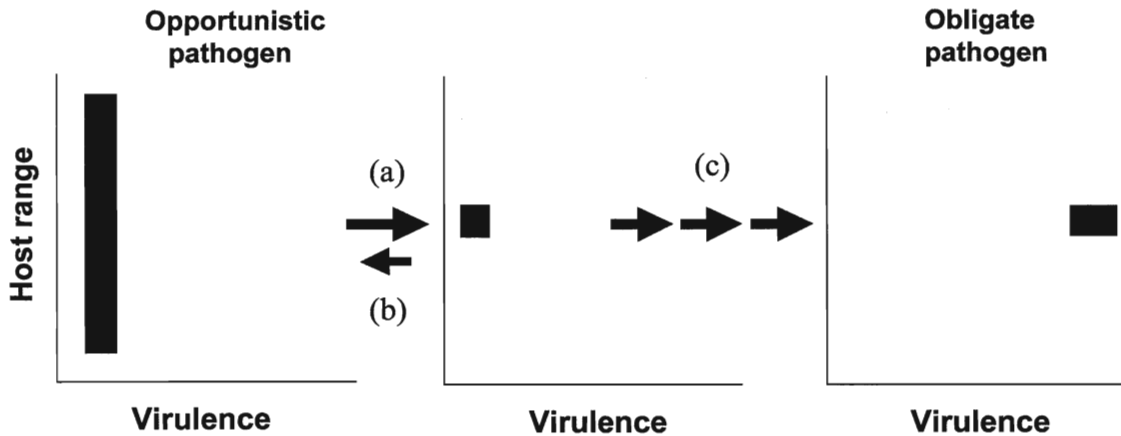


Figure 4-6: A hypothetical model to explain host restriction of an opportunistic pathogen through to host specialization and increased virulence as an obligate pathogen. The opportunistic pathogen exhibits broad host range but relatively low virulence. The obligate pathogen exhibits a narrow host range but relatively higher virulence. (a) Host restriction that is associated with a single gene mutation and auxotrophy. (b) Spontaneous reversion of that mutation restores broad host range. (c) Restricted host range applies selection pressure to the pathogen, resulting in the evolution of host specialization and increased virulence.

further adaptation towards increased virulence. This hypothesis recognizes that an essential prerequisite to pathogenicity is the capacity of the host to provide suitable nutrients for the pathogen. The host must satisfy the nutritional requirements of the pathogen in order for the latter to proliferate and cause disease (Garber 1956). Recent reviews highlight the importance of host-microbe interactions in the production of tissue damage and disease, recognizing that host nutritional status and the ability of a microbe to survive and replicate in a given host are important aspects of pathogenicity (Casadevall & Pirofski 1999; Casadevall & Pirofski 2001).

In many cases, the host is capable of providing nutrients for the growth of an auxotrophic strain of a pathogen such that the microbe retains its pathogenicity and does not exhibit a decrease in virulence. For example, biochemical mutants of *Klebsiella pneumoniae* deficient in the synthesis of one amino acid, threonine, tyrosine, methionine, leucine, or histidine, were as virulent as the wild type toward mice (Garber et al. 1952). The facultative bacterial pathogen *Listeria monocytogenes* auxotrophic for uracil, phenylalanine, glycine, or proline displayed virulence and growth rates similar to the parental strain when bioassayed against mice (Marquis et al. 1993). Similarly, cysteine or methionine auxotrophs of a variety of pathogens retain pathogenicity towards their hosts. Methionine auxotrophs of *Pseudomonas syringae* pv. *glycinea* retain pathogenicity toward soybeans (Thomas & Leary 1980), and methionine or cysteine-methionine auxotrophs of *Candida albicans* show no decrease in virulence toward mice (Manning et al. 1984). With respect to survival in mice, methionine auxotrophs of *Mycobacterium bovis* show no attenuation compared to the wild type (Wooff et al. 2002).

It follows from this explanation of the role of nutrition in pathogenicity that host specificity of obligate pathogens may exist in part because the host is a source of adequate nutritional supplementation (Garber 1960). Indeed, many obligate pathogens exhibit very specific nutrient requirements that must be supplied by the host in order for pathogenicity to occur, including the completion of the pathogen lifecycle. *Rickettsia prowazekii*, the causative agent of louse borne typhus, requires serine, glycine, and proline from the host (Austin et al.

1987; Austin & Winkler 1988), while *Eubacterium suis*, an obligate bacterial pig pathogen causing pyelonephritis and cystitis, utilizes a multitude of carbohydrates, peptides, and vitamins from its host (Wegienek & Reddy 1982). *Yersinia pestis*, the causative agent of plague, possesses a phenotype devoid in ability to synthesize many vitamins, amino acids, and enzymes. As a consequence, *Y. pestis* cannot survive outside its mammalian or flea hosts. Comparison of the *Y. pestis* genome with that of a closely related, free-living, facultative pathogen *Y. pseudotuberculosis* implies that their divergence involved host-range restriction accompanied by metabolic gene inactivation in *Y. pestis* (Hinnebusch 1997).

These studies indicate that at least part of an obligate pathogen's adaptation and restriction to a specific host is correlated with loss of a biosynthetic component concomitantly supplemented by the host. The derivation of the auxotrophic strain *Af6982con^{ins}* in this study also implies a direct association between loss of a biosynthetic component and host restriction. Furthermore, this may be the first step in the emergence of infectious diseases because restriction of the pathogen to the host provides the opportunity for further adaptation via selection, involving the acquisition of virulence factors by spontaneous mutation, recombination, or horizontal transmission.

Chapter V:

Comparative sequence and expression analysis of APS kinase and PAPS reductase: the search for the genetic cause of the insect-host restriction of an *Aspergillus flavus* cysteine/methionine auxotroph

Abstract

In order to study pathogen evolution, we developed a pathogen-host model system amenable to scientific experimentation employing the opportunistic fungus *Aspergillus flavus* and the insect host *Galleria melonella* larvae. During the serial propagation of *A. flavus* 6982 through the insect host, we isolated a strain that displayed properties of an obligate insect pathogen. Initially characterized by a decreased capacity to grow and/or produce conidia on artificial media, this strain also demonstrated a decreased host range since, unlike the wild type, it failed to infect and/or conidiate on plants. This strain was designated *Af6982con^{ins}* because it continued to infect and adequately conidiate on insects despite its deficiencies on agar media or plants. Moreover, these properties of obligate insect pathogenesis were associated with cysteine/methionine auxotrophy, presumably due to a single gene mutation, rendering it unable to convert sulfate to sulfite. (Scully & Bidochka (2006) *Microbiology* 152: 223-232.) Although the APS kinase and PAPS reductase genes were successfully amplified and sequenced from *Af6982* and *Af6982con^{ins}*, no nucleotide sequence alterations were detected, and RT-PCR showed no difference in gene expression. It is likely that the inability of *Af6982con^{ins}* to reduce sulfate to sulfite stems from a mutation in a gene ancillary to APS kinase and/or PAPS reductase but essential for sulfate assimilation. Isolating this mutation would illuminate the types of genetic changes that contribute to pathogen evolution.

Introduction

Pathogen evolution is an essential component of the emergence of devastating novel infectious diseases that plague humanity. Theoretically, virulent facultative and obligate pathogens evolve from opportunistic pathogens via genetic change and concomitant selection in a host environment (Scheffer 1991). In order to study pathogen evolution, we have developed a pathogen-host model system involving infections of the opportunistic fungus *Aspergillus flavus* in the insect-host *Galleria mellonella* larvae (Scully and Bidochka 2005). Insects such as *G. mellonella* larvae mount an immune response that is similar to the mammalian innate immune response and demonstrate similar relative susceptibilities to certain opportunistic pathogens as mammals (Scully & Bidochka 2006b), justifying their use as a model host for the study of pathogen evolution. *A. flavus* is an opportunistic fungus of plants, insect, mammals, and humans (Yu et al. 2005) and as such is hypothetically capable of evolving both increased virulence and host specialization indicative of pathogen evolution.

Using this fungal-insect model system, we isolated a strain of *A. flavus* that demonstrated characteristics of an obligate insect pathogen, namely difficulty growing and/or producing conidia on artificial agar media and a restricted host range as it failed to infect and/or conidiate on plant hosts but continued to conidiate on insects at the completion of the pathogenic cycle (Scully & Bidochka 2006a). Furthermore, growth on minimal media required supplementation with either cysteine or methionine, and the rate of recovery of spontaneous revertants indicated that the phenotype was the result of a single gene mutation. Thus, the insect-host restriction appeared to stem from a nutrient deficiency caused by a single gene mutation instigating a cysteine/methionine auxotrophic phenotype (Scully & Bidochka 2006a).

Cysteine and methionine are the only two amino acids that contain sulfur. Consequently, their biosynthetic pathways are interconnected. The primary mode of cysteine synthesis involves the condensation of O-acetylserine with a sulfide, however, an alternate pathway also exists whereby O-acetylhomoserine combines with H₂S to form homocysteine, which is subsequently

converted to cystathionine and then cysteine (Marzluf 1997; Paszewski & Grabski 1974) (Figure 5-1). Methionine is generated from homocysteine by the addition of a methyl group by homocysteine methyl-transferase (Thomas & Surdin-Kerjan 1997) (Figure 5-1). *Aspergillus* spp. are capable of interconverting between cysteine and methionine through the intermediaries cystathionine, homocysteine, S-adenosylhomocysteine (SAH), and S-adenosylmethionine (SAM) (Marzluf 1997) (Figure 5-1).

Common to the synthesis of both cysteine and methionine is the sulfate assimilation pathway whereby fungi absorb sulfate from the environment and reduce it to sulfide which is eventually incorporated into cysteine or methionine. Following the uptake of sulfate by a sulfate permease (Arst 1968; Pilsyk et al. 2007), it is activated by ATP sulfurylase (adenosyl triphosphate sulfurylase) to form adenosine 5'-phosphosulfate (APS) (Borges-Walmsley et al. 1995; Schierova et al. 2000; Thomas & Surdin-Kerjan 1997). Subsequent phosphorylation is accomplished by APS kinase (adenosyl phosphosulfate kinase) to yield 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Clarke et al. 1997; Thomas & Surdin-Kerjan 1997). The sulfate of PAPS is reduced by PAPS reductase (phosphoadenosyl phosphosulfate reductase) to form sulfite (Borges-Walmsley et al. 1995; Schierova et al. 2000; Thomas & Surdin-Kerjan 1997), which is further reduced by sulfite reductase forming sulfide (Thomas & Surdin-Kerjan 1997) that is incorporated into cysteine or methionine (Figure 5-1).

Since the relatively frequent reversion rate suggested that the cysteine/methionine auxotrophy of the insect-restricted strain of *A. flavus* was presumably due to a single mutation event, the defective gene likely resided in an enzyme of the sulfate assimilation pathway common to both cysteine and methionine. Outside the sulfate assimilation pathway, only a double mutation would accomplish auxotrophy overcome by either cysteine or methionine supplementation (Paszewski & Grabski 1974). Indeed, biochemical analysis indicated that the *A. flavus* cysteine/methionine auxotroph was unable to enzymatically convert sulfate to sulfite

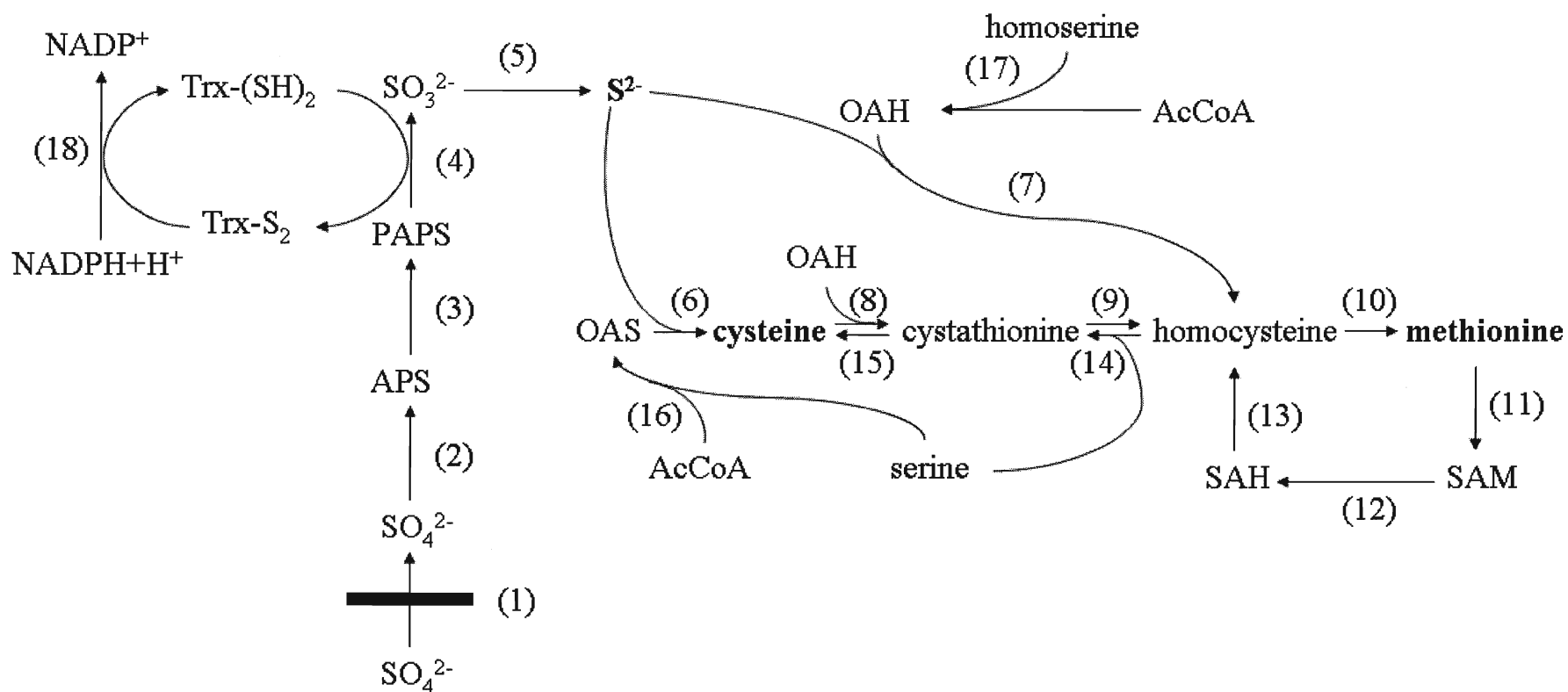


Figure 5-1: Sulfate assimilation and biosynthesis of cysteine and methionine in *A. nidulans*. (OAS) O-acetyl serine, (OAH) O-acetyl homoserine, (SAM) S-adenosylmethionine, (SAH) S-adenosylhomocysteine, (PAPS) 3'-phosphoadenosine 5'-phosphosulfate, (APS) adenosine 5'-phosphosulfate, (Trx) thioredoxin, (1) sulfate permease, (2) ATP sulfurlyase, (3) APS kinase, (4) PAPS reductase, (5) sulfite reductase, (6) cysteine synthase, (7) homocysteine synthase, (8) cystathionine γ -synthase, (9) cystathionine β -lyase, (10) methionine synthase, (11) S-adenosylmethionine synthase, (12) various methyltransferases, (13) S-adenosylhomocysteine hydrolase, (14) cystathionine β -synthase, (15) cystathionine γ -lyase, (16) serine acetyltransferase, (17) homoserine acetyltransferase, (18) thioredoxin reductase (Thomas & Surdin-Kerjan 1997; Marzluf 1997; Porque et al. 1970).

during sulfate assimilation. Because ATP sulfurlyase was functional, the genetic defect appeared to involve either APS kinase or PAPS reductase activity (Scully & Bidochka 2006a). The purpose of this study was to determine the genetic source of the cysteine/methionine auxotrophy by performing comparative sequence and expression analysis on APS kinase and PAPS reductase of the cysteine/methionine auxotroph of *A. flavus* and its ancestral wild type strain. Isolating this genetic mutation would further the understanding of emerging diseases, since it would illuminate the types of genetic changes that can contribute to pathogen evolution.

Materials and Methods

Fungal strains and culture conditions

A. flavus 6982 (*Af*6982) was obtained from the University of Alberta Microfungus Collection and Herbarium (Edmonton, AB). *A. flavus* 6982con^{ins} (*Af*6982con^{ins}) is cysteine/methionine auxotroph derived from *Af*6982. Compared to *Af*6982, *Af*6982con^{ins} displays a severe reduction in conidial production on potato dextrose agar (PDA) and insect-host restriction since it is pathogenic towards insects, including adequate conidiation on the cadavers, but fails to infect and/or conidiate on various plants (Scully and Bidochka 2006a). *A. flavus* 46114 is a methionine auxotroph obtained from the American Type Culture Collection (Manassas, VA) (Papa 1980). All strains were grown on potato dextrose agar (PDA) (Difco) at 30°C.

YADE

Fungi (5×10^4 conidia) were inoculated into 50ml of YPD broth (0.2% yeast extract, 1% peptone, 2% dextrose) and incubated for 3 d, 250rpm at 27°C. The cultures were filtered from the liquid media through sterile Büchner funnels and crushed in liquid nitrogen using a mortar and pestle. DNA was extracted using the Qiagen DNeasy tissue extraction kit (Qiagen) according to the manufacturer's protocol.

The APS kinase and PAPS reductase genes were amplified using degenerate primers based on the sequences reported for *Emmericella nidulans*, *Aspergillus fumigatus*, and *Aspergillus terreus* (NCBI Acc. # XP_752447, CAA70089, XP_658798, XP_754952, AAF28889, P56859, XP_662374). All PCR reactions utilized the HotStarTaq DNA polymerase kit (Qiagen) and primers from Sigma. The reaction mixture for APS kinase amplification contained 0.25µl HotStarTaq (Qiagen), 5µl of 10X PCR buffer (Qiagen), 10µl of 5X Q-solution (Qiagen), 200µM of each dNTP, 3µl DNA, 0.9µM DAPS-F, and 0.9µM DAPS-R (Table 5-1) in 50µl. The PCR amplification conditions were 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 45°C for 60 s, and 72°C for 60 s, followed by 72°C for 10 min. The PCR reaction mixture for PAPS reductase amplification was identical except that 0.45µM each of DPAPS-F and DPAPS-R (Table 5-1) were substituted for DAPS-F and DAPS-R. The resulting PCR products were subjected to agarose gel electrophoresis, excised from the gel, and purified using the Qiagen Gel Extraction Kit (Qiagen). The purified products were sequenced by the York University Core Molecular Biology and Sequencing Facility.

The YADE technique (Y-shaped adaptor dependent extension) (Xiao et al. 2007) required digesting *Af6982* and *Af6982con^{ins}* DNA with various digestion enzymes, ligating the digested DNA to a Y-shaped YADE adaptor (see below), and amplifying the sequence with a linear PCR amplification followed by an exponential PCR amplification. Primers for YADE were designed based on the partial sequences amplified using the degenerate primers (Table 5-1). The DNA was digested according to the manufacturer's instruction (New England Biolabs). Then, 1µl of digested DNA (~10ng) was incubated with 400 U T4DNA ligase (New England Biolabs), 1µl of 10X ligation buffer (New England Biolabs), and 1µl adaptor (0.068 µg/µl) (Table 5-1) in 10µl for 16 h at 16°C. The linear amplification mixture contained 1 U HotStarTaq DNA polymerase (Qiagen), 1µl of the ligation products, 2.5µl of 10X PCR buffer (Qiagen), 1µl primer (5µM; Table 5-1), 0.5mM MgCl₂, and 0.2mM of each dNTP in a total volume of 25µl and

Table 5-1: List of primers and oligonucleotides used to amplify, sequence, and analyze the expression of APS kinase and PAPS reductase of *A. flavus*

Experiment	Primer	Sequence (5'-3')
Degenerate PCR		
APS kinase	DAPS-F	CTCACAATHHTGGYTACNNGG*
	DAPS-R	ATCYCYCTTYTCNGCNACYTC*
PAPS reductase	DPAPS-F	TTCACCAARCCWCAYCTNCARTT*
	DPAPS-R	GGCGTACTTKGAVCGNGGRTRTG*
YADE	YADEE	CGGTAGGATCCCGCAGAACGA
5'APS kinase	<i>XbaI</i> -adapter	CGGTAGGATCCCGCAGAACGACGGCCAG/ CTGGCCGTCCAAGACGC
	APSL5	CGAGGTGATGGCGATAGAGG
	APSE5	CGTCGAGACGGTAGGCGTGG
3'APS kinase	<i>EcoRV</i> -adapter	CGGTAGGATCCCGCAGAACGACGGCCAG/ CTGGCCGTCCAAGACGC
	APSL3	GACCGCAACGAGAACATCCG
	APSE3	CCTCTATCGCCATCACCTCG
5'PAPS reductase	<i>XbaI</i> -adapter	CGGTAGGATCCCGCAGAACGACGGCCAG/ CTGGCCGTCCAAGACGC
	PAPSL5	GATGAGGTCGACCATCTGAGG
	PAPSE5	TCTGGAAAAGGTGAGGGAGTG
3'PAPS reductase	<i>SmaI</i> -adapter	CGGTAGGATCCCGCAGAACGACGGCCAG/ CTGGCCGTCCAAGACGC
	PAPSL3	ACATCAAAGACAACGATGTGCC
	PAPSE3	GCGGCTACAAGAGCATTTGGTG
High fidelity PCR		
APS kinase	HFAPS-F	CTCCATCCGCAGCCACTAAC
	HFAPS-R	CATATCTAGTCCGCAAATATCCGC
PAPS reductase	HFPAPS-F	CTATCCTACATCATCTCACGC
	HFPAPS-R	AACTCCCAATCCCAAACGCC
RT-PCR		
APS kinase	RTAPS-F	AACATCACCTTCCATGCCAGC
	RTAPS-R	AACTCCGAAATCTTACCCGCC
PAPS reductase	RTPAPS-F	CTGATACCTACCCCTCCCACG
	RTPAPS-R	CCGTTTCATCCTCGTTTTCCTTG
β -tubulin	RTBTUB-F	AGGGTAACCAAATAGGTGCCG
	RTBTUB-R	GGTCGTTTCATGTTGCTCTCA

* H = A + T + C

Y = C + T

N = A + T + C + G

R = A + G

W = A + T

K = G + T

was cycled 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min, followed by 72°C for 2 min. The exponential PCR reaction contained 1 U HotStarTaq DNA polymerase (Qiagen), 1µl linear PCR product, 2.5µl 10X PCR buffer (Qiagen), 1µl YADEE (5µM, Table 5-1), 1µl primer (5µM, Table 5-1), 0.5mM MgCl₂, and 0.2mM dNTPs in 25µl and was cycled 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 150 s, followed by 72°C for 10 min. The exponential PCR products were electrophoresed on 1% agarose gels, excised and purified from the gels, and sequenced as described above.

The 5' end of the APS gene was obtained following digestion with *Xba*I, ligation to the *Xba*I-adaptor, linear amplification with primer APSL5, and exponential amplification with primers APSE5 and YADEE, while the 3' end of the gene was amplified using YADE in conjunction with *Eco*RV, *Eco*RV-adaptor, APSL3, and APSE3/YADEE. Similarly, the 5' end of the PAPS reductase gene utilized *Xba*I, *Xba*I-adaptor, PAPSL5, and PAPSE5/YADEE, while the 3' end required *Sma*I, *Sma*I-adaptor, PAPSL3, and PAPSE3/YADEE (Table 5-1).

The YADE adaptors were constructed by annealing a short chain oligonucleotide with a long chain oligonucleotide to form a Y-shaped adaptor (Table 5-1). The short chain was phosphorylated by incubating 1.36µg of the short chain with 20 U T4 polynucleotide kinase (New England Biolabs), 2µl of 10X T4 polynucleotide kinase buffer (New England Biolabs), and 1 mM ATP in a total of 20µl at 37°C for 30 min followed by 72°C for 10 min. Then, 4µl of 10X annealing buffer (1M NaCl, 0.1M Tris·Cl pH 7.0, 0.01M EDTA) and 1.36µg of the long chain were added to the phosphorylated short chain solution, and the final volume was adjusted to 40µl. The reaction mixture was heated at 65°C for 10 min and cooled slowly to room temperature to facilitate annealing between the two chains.

Once the *A. flavus* APS kinase and PAPS reductase sequences were obtained, each gene was amplified using a high-fidelity DNA polymerase and sequenced. PCR reactions were carried out using AccuTaq (Sigma) and HFAPS-R/HFAPS-R or HFPAPS-F/HFPAPS-R primer

combinations. The reaction mixtures contained 1µl AccuTaq (Sigma), 5µl 10X PCR buffer (Sigma), 0.5mM of each dNTP, and 1µl of each primer (20uM) in a total of 50µl. The PCR conditions were 96°C for 30 s, followed by 30 cycles of 94°C for 10 s, 62°C (APS kinase) or 55°C (PAPS reductase) for 30 s, and 68°C for 2 min, followed by 68°C for 30 min. The products were electrophoresed, purified, and sequenced as described above.

Alignment and cluster analysis

The APS kinase and PAPS reductase genes of *Af6982* and *Af6982con^{ins}* were aligned with sequences of other *Aspergillus* spp. obtained from GenBank using BioEdit. After constructing similarity matrices based on the nucleotide sequences of the coding regions in BioEdit, cluster analysis was performed by unweighted pair-group method using arithmetic averages (UPGMA) using NYSTS-pc version 2.1 (Exeter software 1986-2002).

RT-PCR

For total RNA isolation, 5×10^4 conidia were inoculated into 50ml YPD broth and incubated at 27°C, 250rpm for 3 d. The mycelia were filtered from the YPD broth through a sterile Büchner funnel, washed thoroughly with sterile dH₂O, and transferred to 50ml Czapek Dox broth (Difco). After 2 d incubation at 27°C, 250rpm, the fungi were again filtered from the media and crushed in liquid nitrogen for RNA isolation. RNA from *Af6982*, *Af6982con^{ins}*, and *Af46114* was isolated using the RNeasy Miniprep kit (Qiagen). RT-PCR was performed using the One-Step RT-PCR kit (Qiagen) with reaction mixtures containing 1µl RNA, 2µl enzyme solution, 3µl each of either RTAPS-F/RTAPS-R (10uM/ Table 5-1), RTPAPS-F/RTPAPS-R (10µM; Table 5-1), or RTBTUB-F/RTBTUB-R (10µM; Table 5-1) primers, 10µl of 5X Q-solution, 2µl of the dNTP mixture, and 10µl of 5X RT-PCR buffer in a total of 50µl, and the RT-PCR conditions were 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, followed by 72°C for 10 min. RT-PCR products were electrophoresed on a 1% agarose gel.

Results

Comparative sequence analysis of APS kinase and PAPS reductase

The complete DNA sequences of APS kinase and PAPS reductase were obtained from *Af*6982 and *Af*6982con^{ins} and submitted to Genbank (accession nos. EU016184, EU016185, EU016186, EU016187). Upon comparison, no nucleotide differences were detected between *Af*6982 and *Af*6982con^{ins} for either the APS kinase or the PAPS reductase genes.

The open reading frames (ORF) of these sequences have been aligned with the corresponding sequences from *A. fumigatus*, *E. nidulans*, *A. terreus*, and *A. clavateus* (see Appendix G).

Dendrograms constructed from the nucleotide sequences of the coding regions of the APS kinase and PAPS reductase genes of *Af*6982, *Af*6982con^{ins}, and other *Aspergillus* spp. show 69-78% and 73-80% similarity between the APS kinase and PAPS reductase coding sequences of *Af*6982/*Af*6982con^{ins} and other *Aspergillus* sp. respectively (Figure 5-2).

Comparative expression analysis of APS kinase and PAPS reductase

In order to determine if the APS kinase and PAPS reductase genes of the sulfate assimilation pathway were expressed in both *Af*6982 and *Af*6982con^{ins}, RNA was isolated during growth in minimal media containing sulfate. Growth in this media ensures that the enzymes of the sulfate assimilation pathway are upregulated. RT-PCR indicated that both the APS kinase and PAPS genes were transcribed by *Af*6982 and *Af*6982con^{ins} as well as by the methionine auxotroph *Af*46114 (Figure 5-3).

Discussion

During the serial propagation of the opportunistic fungus *A. flavus* 6982 through the insect host *G. mellonella* larvae, we recovered a strain of *A. flavus* with some properties of an obligate insect pathogen. This strain, designated *Af*6982con^{ins}, proved difficult to grow outside of its insect host to which it remained pathogenic. It failed to grow and/or conidiate on artificial culture media and was unable to infect and/or conidiate sufficiently on plant hosts. Further analysis indicated that it was a cysteine/methionine auxotroph with an inability to convert sulfate

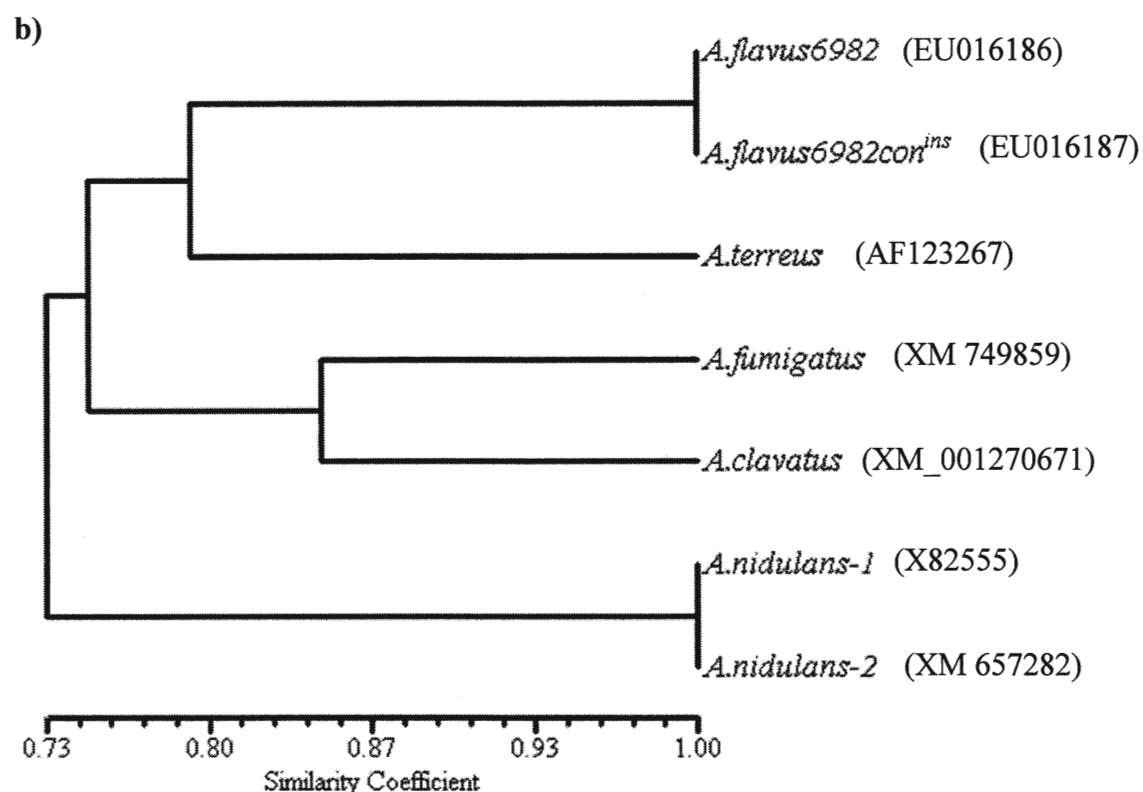
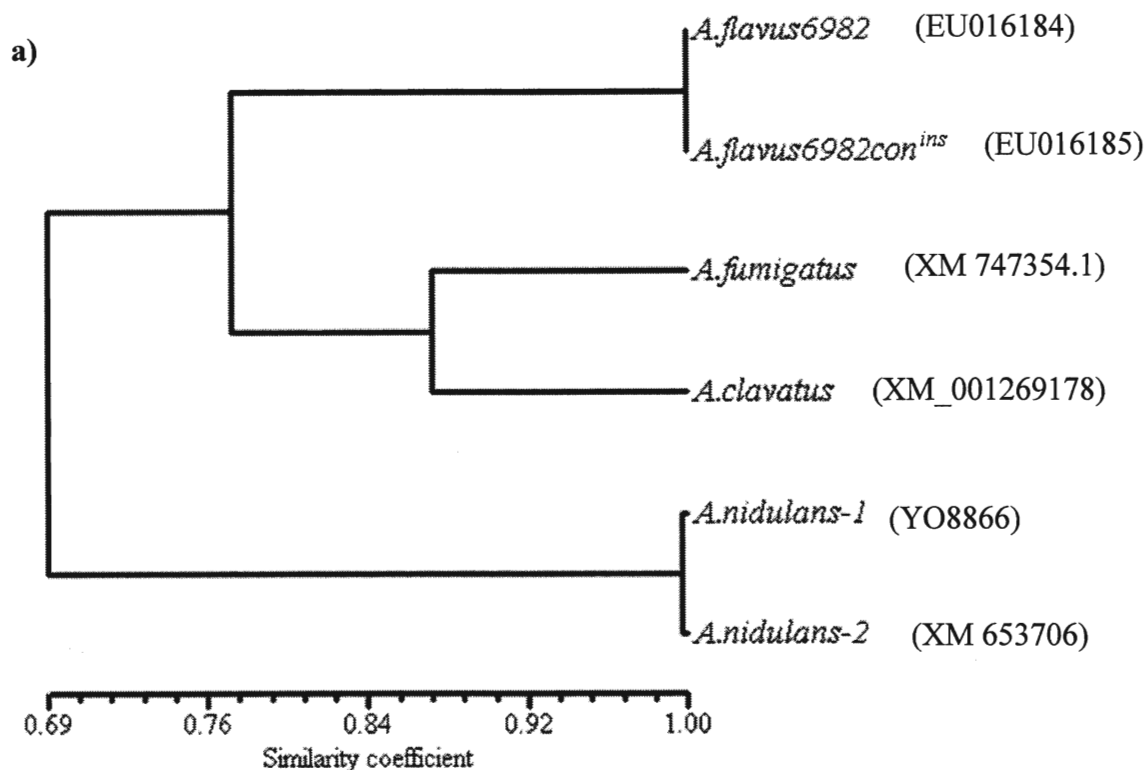


Figure 5-2: UPGMA cluster analyses derived from similarity matrices generated from simple matching coefficients based on the nucleotide coding sequences of (a) APS kinase and (b) PAPS reductase of *Aspergillus* sp. Scales represent simple matching coefficients. The GenBank accession number is listed in parenthesis following the species name.

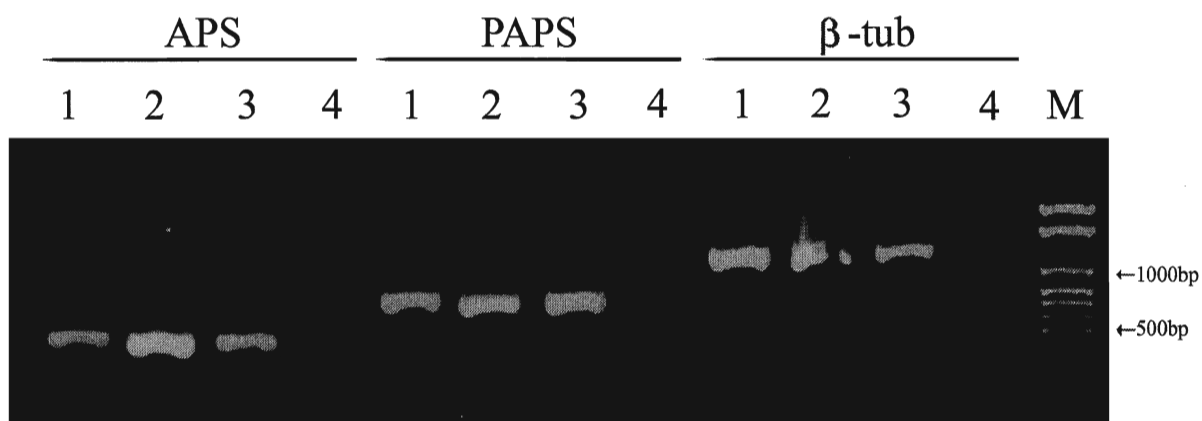


Figure 5-3: RT-PCR analysis of expression of APS kinase (APS) and PAPS reductase (PAPS) in *Af6982* (1) *Af6982con^{ins}* (2) and *Af46114* (3) with ddH₂O (4) as a negative control. β-tubulin served as a positive control.

to sulfite, presumably due to a single gene mutation. Thus, the appearance of cysteine/methionine auxotrophy was correlated with properties of obligate insect pathogenesis, providing a hypothetical model for the evolution of obligate pathogenesis whereby a nutrient deficiency restricts a pathogen to a specific host and provides the selection pressure for an increase in virulence (Scully & Bidochka 2006a).

Biochemical analysis revealed that *Af6982con^{ins}* retained a deficiency in the sulfate assimilation pathway, specifically an inability to reduce sulfate to sulfite. However, since ATP sulfurylase was functional, the defect was manifest as a lack of APS kinase or PAPS reductase activity (Scully & Bidochka 2006a). Although we sequenced the complete APS kinase and PAPS reductase genes of *A. flavus*, no nucleotide alterations were detected between *Af6982* and *Af6982con^{ins}*. Furthermore, expression analysis using RT-PCR indicated that the genes were transcribed in both strains. Apparently, the cause of the cysteine/methionine auxotrophy of *Af6982con^{ins}* was not due to a genetic defect in either the APS kinase or the PAPS reductase genes.

Because the APS kinase and PAPS reductase genes appeared to be intact and functional in *Af6982con^{ins}*, the inability of this strain to convert sulfate to sulfite was probably due to a mutation in a gene ancillary to the APS kinase/PAPS reductase steps of the sulfate assimilation pathway. In *Saccharomyces cerevisiae*, separate mutations in at least five different loci abolished PAPS reductase activity. In addition to the PAPS reductase gene (*MET16*), these loci included a positive-acting regulatory gene (*MET4*), genes involved in siroheme synthesis (*MET1*, *MET8*), and the homocysteine synthase gene (*MET25*) (Thomas et al. 1990; Thomas & Surdin-Kerjan 1997). The reduction of PAPS to sulfite also requires thioredoxin and thioredoxin reductase in conjunction with PAPS reductase (Porque et al. 1970; Russel et al. 1990), since reduced thioredoxin serves as an electron donor which PAPS reductase uses during the sulfate reduction reaction (Berendi et al. 1995). These represent at least a partial list of possible genes that could be disrupted in *Af6982con^{ins}*, leading to an inability to convert sulfate to sulfite.

The recent completion of the *A. flavus* genomic library (Payne et al. 2006) will facilitate the screening of *Af6982con*^{ins} to elucidate the genetic source of the mutation that caused concomitant cysteine/methionine auxotrophy and insect-host restriction. Using this library, primers can easily be designed to amplify and sequence the various accessory genes involved in the sulfate assimilation pathway. This knowledge will advance the understanding of emerging diseases since it will reveal the types of genetic mutations that contribute to pathogen evolution.

Chapter VI:

Pathogen auxotrophy limits host range and increases virulence factor production in a model pathogen-host system.

Abstract

In order to study pathogen evolution, we used a model system whereby the opportunistic fungus *Aspergillus flavus* was serially propagated through *Galleria mellonella* larvae, yielding a cysteine/methionine auxotroph of *A. flavus* with properties of an obligate insect pathogen, namely host restriction without a decrease in virulence (Scully & Bidochka (2006) *Microbiology* 152: 223-232). Here, we report that besides host restriction, the auxotroph exhibited enhanced virulence factor production, another notable aspect of pathogen evolution. While germination of the auxotroph was delayed and hyphal growth rate was slower than the wild-type on “insect” media, secretion of extracellular proteases, important virulence factors in insect pathogenesis, was enhanced. Presumably, higher protease production compensated for the depressed growth of the auxotroph such that virulence toward *G. mellonella* was maintained. In the wild-type, protease production was derepressed during carbon (glucose), nitrogen (nitrate), or sulfate deprivation. If all three were present protease production was vastly reduced. However, in the cysteine/methionine auxotroph, protease production was deregulated in complete media indicating a deficiency in sulfate assimilation and accounting for the enhanced protease secretion in “insect” media. Although there were no differences in conidial adherence, appressoria formation, cuticular penetration, or virulence, the wild-type out-competed the auxotroph during competition bioassays with insect larvae. In this model system, the biosynthetic deficiency that mediated host restriction and increased virulence factor (protease) production highlights the interplay between nutrition and genome decay in pathogen evolution.

Introduction

Concern about emerging human diseases has recently escalated because of the number of pathogens that are infecting humans with increasing frequency (Taylor et al. 2001).

Understanding the factors that contribute to the evolution of a successful pathogen would allow increased vigilance against potential emerging infectious agents. Theoretically, virulent, host-specific facultative and obligate pathogens evolved from less virulent, opportunistic pathogens (Scheffer 1991). Accordingly, pathogen evolution involves both host specialization and an increase in virulence through horizontal virulence factor acquisition and/or de novo mutations that modulate existing virulence components. An often overlooked component of pathogen evolution is genome decay, which can be selectively neutral or adaptive. For example, obligate pathogens may lose biosynthetic capacity for a component that can be acquired from their hosts (Maruelli 2007; Wren 2003).

In order to study pathogen evolution, we employed a model pathogen-host system involving the serial propagation of the opportunistic fungus *Aspergillus flavus* through the insect host *Galleria mellonella* larvae (Scully & Bidochka 2005). *A. flavus* and *G. mellonella* are ideal species for such a model system. *A. flavus* is an opportunistic pathogen that exhibits broad host range yet with low virulence (Yu et al. 2005) and possesses the possibility of evolving host specialization and increased virulence. *G. mellonella* larvae are susceptible, easily manipulated hosts available in large numbers. Furthermore, various aspects of the insect innate immune system and the infection response render pathogen studies using insects as hosts applicable to human pathogenic microbes (Scully & Bidochka 2006b).

During serial passage, we isolated a strain of *A. flavus* demonstrating a severe diminution of conidial production or complete lack of growth on microbiological media, while maintaining adequate conidial production at the culmination of the pathogenic cycle on insects. Although this strain remained pathogenic to insects with no decrease in virulence toward *G. mellonella* larvae compared to the wild-type, it failed to infect or conidiate sufficiently on plants.

Essentially, this strain of *A. flavus* exhibited characteristics of an obligate insect pathogen (Scully & Bidochka 2006a). Biochemical analysis characterized it as a cysteine/methionine auxotroph, deficient in the ability to convert sulfate to sulfite, leading us to hypothesize that this nutrient deficiency, fulfilled best during growth on insects, accomplished host restriction, a notable feature of pathogen evolution. We postulated that host restriction due to a biosynthetic deficiency may be an important step of obligate pathogen evolution since it may provide the physiological backdrop for the selection pressure required to develop further host specialization and increased virulence (Scully & Bidochka 2006a).

Here, we report a pleiotrophic effect of cysteine/methionine auxotrophy on virulence factor production. When grown on media mimicking conditions experienced during insect pathogenesis, the insect-restricted auxotroph exhibited enhanced protease production. Extracellular proteases, particularly subtilisin-like proteases, are important virulence factors secreted by facultative insect pathogens to degrade the proteinacious insect cuticle, allowing insect infection (St. Leger 1995). In the insect-restricted strain, the enhanced protease secretion was presumably a byproduct of cysteine/methionine auxotrophy since fungi secrete extracellular proteases in response to carbon, nitrogen, or sulfur starvation (Cohen 1973; Hanson & Marzluf 1975; Srinivasan & Dhar 1990). Enhanced protease secretion presumably offset the slow growth of the auxotroph during insect pathogenesis, allowing indistinguishable cuticular penetration and virulence toward *G. mellonella* larvae. These results highlight a biosynthetic nutrient deficiency as a mechanism of both host restriction and enhanced virulence factor production during pathogen evolution.

Materials and Methods

Fungal strains and culture conditions

A. flavus strain 6982 (*Af*6982) was obtained from the University of Alberta Microfungus Collection and Herbarium. *Aspergillus flavus* 6982con^{ins} (*Af*6982con^{ins}) was derived from *Af*6982 as previously described (Scully & Bidochka 2006a). *Af*6982con^{ins} is an insect-restricted

strain of *A. flavus*, displaying certain characteristics of an obligate insect pathogen. Although *Af6982con^{ins}* exhibits a drastic reduction in conidial production when grown on artificial media (PDA) and an inability to infect and/or conidiate sufficiently on various plant species, it retains its ability to infect insects and adequately conidiates on the surface of insect cadavers. Furthermore, *Af6982con^{ins}* is a cysteine/methionine auxotroph. All strains were maintained at 30°C on Petri dishes containing 10ml of PDA (Potato Dextrose Agar, Difco).

Biomass determination

Fungal strains were subcultured using a sterile toothpick onto Petri dishes containing 10ml of either 1% cuticle agar or synthetic *Galleria* media (SGM) agar. The cuticle was obtained from *Locusta sp.* courtesy of R. Meldrum Robertson (Queens University, ON) as described by Bidochka and Khachatourians (1990). The SGM was prepared as described by Dunphy and Webster (1986). Colonies were photographed following 7 d incubation at 30°C. To determine the amount of fungal biomass on the SGM media, the Petri dishes were immersed in boiling water to liquefy the agar. The fungal colonies were rinsed thoroughly to remove the agar media, dried, and weighed. Biomass was determined as a mean of three replicates.

Germination and hyphal growth rate

Petri dishes containing 10ml of 1% cuticle agar or SGM agar were inoculated by spread-plate method with 1×10^4 *Af6982* or *Af6982con^{ins}* conidia and incubated at 30°C. At 1 h time intervals from 6-12 h following inoculation (4 h time intervals from 10-30 h following inoculation for *Af6982con^{ins}* on 1% cuticle), sections of the agar media were removed from the plates, stained with lactophenol cotton blue, and examined microscopically. For each time interval, 100 conidia were assessed for percent germination and average hyphal length. From these data, GT_{50} (time for 50% of the conidia to germinate) was calculated using Probit analysis with statistical significance determined by a lack of overlap between the 95% confidence intervals. The hyphal growth rate was calculated as $\Delta \log_{10}(\text{average hyphal length (mm)}) / \Delta t$ (h). The data presented represents three trials.

Enzyme assays in insect media

Twenty-five milliliters of 1% cuticle or SGM liquid media were inoculated with conidia to a final concentration of 2×10^6 conidia ml^{-1} and incubated at 30°C and 250rpm for 18 h. The extracellular filtrates were collected by filtering through 0.2 micron sterile filters and stored at -80°C . The filtrates were assayed for subtilisin-like protease activity toward succinyl-(alanyl)₂-prolyl-phenylalanine *p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-NA) (Sigma), and trypsin like activity toward N-benzoyl-phenylalanyl-valyl-arginine *p*-nitroanilide (Bz-Phe-Val-Arg-NA) (Sigma) (St. Leger et al. 1987a). For total protease activity, 100 μl of fungal culture filtrate was incubated with 1mg hide powder azure, 100 μl of 1.0M NaOH-glycine buffer pH 8.5, and 300 μl ddH₂O at 37°C at 180rpm for 3.5 h, and the absorbance of the supernatant was measured at 630nm. Enzyme assays were performed in triplicate. The amount of total protease, subtilisin-like protease, and trypsin-like protease activity produced by *Af6982* and *Af6982con*^{ins} in cuticle or SGM were compared using t-tests ($\alpha = 0.05$).

Enzyme assays in minimal media

Fungi were grown in 100ml YPD broth (2.0% dextrose, 1.0% peptone, 0.2% yeast extract) for 3 d at 30°C at 250rpm after which they were filtered from the YPD broth, washed thoroughly with sterile dH₂O, and ~2g wet weight was transferred to 40ml of one of the following media: minimal media (MM) (0.036% [w/v] KH₂PO₄, 0.093% Na₂HPO₄, 0.10% KCl, 0.06% MgSO₄·7H₂O, 0.15% NaNO₃, 1.0% dextrose, 0.08% elastin), MM lacking a carbon source (dextrose) (MM-C), MM lacking a nitrogen source (NaNO₃) (MM-N), MM lacking a sulfur source (MgSO₄·7H₂O) (MM-S), and MM lacking MgSO₄·7H₂O but supplemented with 10 mM methionine (MM + met). The cultures were incubated for 18 h at 30°C at 250rpm before the extracellular filtrate was collected, filtered through 0.2 micron sterile filters, and assayed for subtilisin-like, trypsin-like, and total protease activity as described above with the assays performed in duplicate.

Isoelectric focusing

The extracellular filtrate from fungal cultures grown for 3 d in YPD and transferred to MM-C, MM-N, MM-S, MM, MM + met, 1% cuticle, and SGM for 18 h at 30°C were frozen and lyophilized. The residual solids were dissolved in 500 µl ddH₂O (4°C), desalted, and concentrated to <50µl by ultrafiltration using Nanosep 10K omega centrifugal devices according to the manufacturer's instructions (Pall). Samples were subjected to analytical isoelectric focusing (pH 3-10) using Model 111 Mini IEF Cell (BioRad) according to the manufacturer's instruction. Total proteolytic activity was detected by gelatin zymography where the IEF gels were overlaid with X-ray film and incubated at 37°C for 30 min or 2 h until the proteases produced zones of clearing in the gelatin layer of the X-ray film. Subtilisin-like protease activity was detected by overlaying the IEF gels with enzyme overlay membranes (EOM) with cellulose acetate membranes infused with synthetic peptide succinyl-(alanyl)₂-prolyl-phenylalanine-7-amino-4-trifluoromethylcoumarin (MP Biomedicals) (Smith 1984). Following incubation at 37°C for 3 h, the EOMs were photographed under UV light with fluorescent white areas indicative of protease activity.

Adherence and appressoria formation

The adherence properties of *Af6982* and *Af6982con^{ins}* were assessed on polystyrene Petri dishes known to mimic the hard, hydrophobic insect cuticular surface (Butt 1987). Conidial suspensions of *Af6982* and *Af6982con^{ins}* were prepared from 7 d old PDA cultures using 5ml of 0.01% Triton X-100. The conidia were washed 3 times with sterile ddH₂O and adjusted to a concentration of 5×10^4 conidia ml⁻¹ in 0.0125% yeast extract. Five milliliters of the suspension was placed on 5.5cm-diameter polystyrene Petri dishes, which were incubated at 30°C for 16 h without movement. The Petri dishes were prepared in duplicate with one of the dishes subjected to a brief jet of water. The percentage adherence was determined by comparing the number of adhered conidia in 15 fields of view of the washed and unwashed dishes. The experiments were performed in triplicate.

The percentage of germinated conidia that formed appressoria on insect cuticle was assessed on isolated *Galleria mellonella* (Peterborough Live Bait, Peterborough, ON) larval cuticles. Cuticles were prepared by removing the heads and dissecting the larvae along the ventral midline. The body cavity contents were removed, and the cuticles were washed thoroughly in sterile dH₂O. Prepared cuticles were inoculated with 5×10^3 conidia, incubated at 30°C for 24 h, stained with 0.01% Calcofluor (Fluorescent Brightener 28, Sigma) (Butt 1987), and viewed and photographed with a Leitz Diaplan microscope with an epifluorescence attachment. Fifty germinated conidia were assessed for percentage appressoria formation. The experiment was performed in triplicate.

Chi-square tests ($\alpha = 0.05$) were employed to determine any statistically significant differences in percentage adherence and appressoria formation.

Competition assay

Sets of 25 larvae of *G. mellonella* (Peterborough Live Bait, Peterborough, Ontario) were topically infected with 5 μ l of 1×10^5 conidia ml⁻¹ suspensions of various ratios (10:0, 9.4:0.6, 8.8:1.2, 8.1:1.9, 7.3:2.7, 6.5:3.5, 5.5:4.5, 4.4:5.6, 3.1:6.9, 1.7:8.3, 0:10) of *Af6982*: *Af6982con*^{ins}. The insects were housed individually in plastic snap cap vials and incubated at 30°C. Upon death, the insects were surface sterilized by washing in 1% NaOCl for 1 minute followed by two 2-min washes with sterile dH₂O, and a moist sterile tissue was added to the vial to encourage conidiation on the cadavers. Cadavers were allowed to conidiate for 5 d before vortexing in 3ml of 0.01% Triton X-100 to obtain a conidial suspension. Using the suspension from each examined insect, approximately 100 conidia were spread-plated on each of 3 PDA Petri dishes and each of 3 Cz agar Petri dishes (Czapek Solution Agar, Difco). *Af6982* colonies form on both media, while *Af6982con*^{ins} colonies form only on PDA. Following incubation at 30°C for 2 d, the colony forming units (CFU) were counted on each plate. T-tests ($\alpha = 0.05$) were performed to determine if the number of CFU on the PDA and Cz media differed significantly. A non-significant difference indicated virtually only *Af6982* CFU, while a significant difference

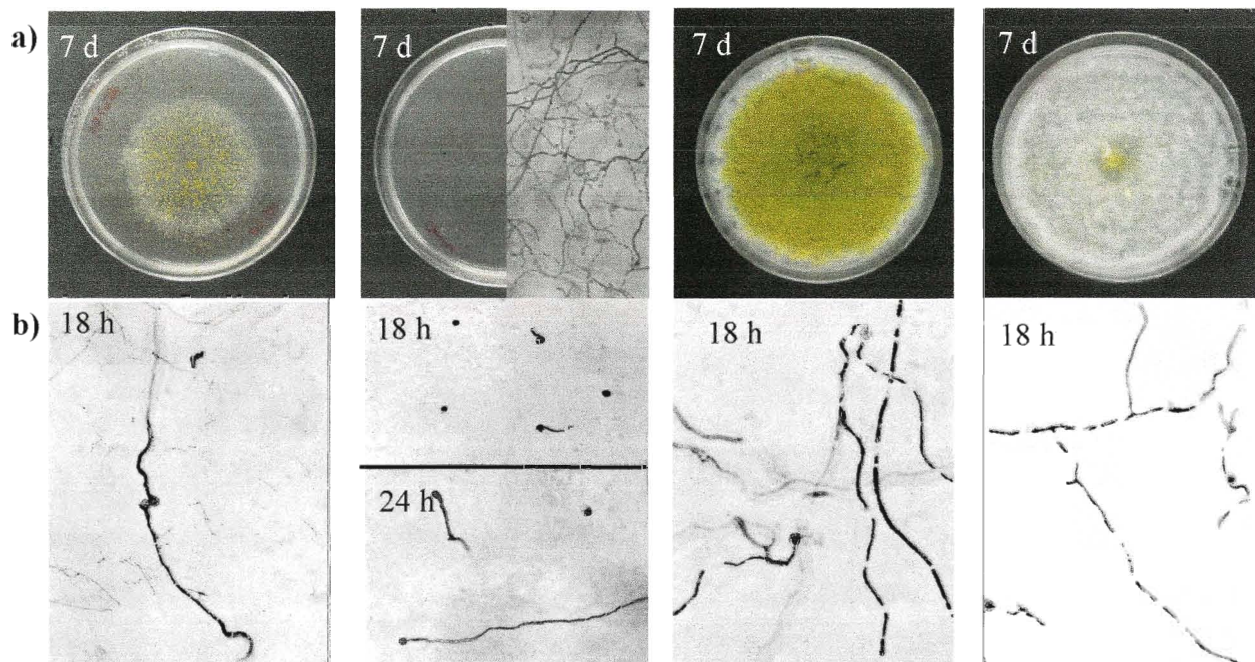
indicated both *Af6982* and *Af6982con*^{ins} CFU. When a significant difference in the number of CFU on the PDA and Cz media was obtained, Chi-square tests ($\alpha = 0.05$) were employed to determine if the proportion of *Af6982* : *Af6982con*^{ins} CFU differed from the ratio in the original inoculum.

In addition as controls, suspensions of various ratios (10:0, 8.8:1.2, 6.3:3.7, 3:7, 0:10) of *Af6982*:*Af6982con*^{ins} were prepared, and ~100 conidia of each suspension were plated on each of 3 PDA Petri dishes and 3 Cz Petri dishes. Following incubation at 30°C for 2 d, the CFU were counted. Chi-square tests ($\alpha = 0.05$) were performed to ascertain whether the number of CFU approximated the ratios of the original suspensions.

Results

Af6982con^{ins} germination and growth is stunted on cuticle media

A. flavus strains *Af6982* and *Af6982con*^{ins} displayed different characteristic growth patterns on 1% cuticle agar and SGM agar media, both of which mimic the nutritional environment encountered during infection of insects. Although *Af6982* grew substantially on 1% cuticle agar after 7 d, *Af6982con*^{ins} produced only microscopic amounts of growth (Figure 6-1a). However, on SGM agar, both *Af6982* and *Af6982con*^{ins} grew robustly (Figure 6-1a), but colony biomass was significantly less for *Af6982con*^{ins} ($0.056\text{g} \pm 0.010$) than for *Af6982* ($0.148\text{g} \pm 0.008$) ($t = 7.145$, $p = 0.002$). Similarly, *Af6982* germinated significantly faster than *Af6982con*^{ins} and exhibited a faster hyphal growth rate on 1% cuticle agar ($t = 7.702$, $p = 0.005$) (Figure 6-1b, Table 6-1, see Appendix H). Conversely, there was no difference in the GT_{50} values when the strains were inoculated onto SGM agar. The hyphal growth rates on SGM agar were statistically different ($t = 3.667$, $p = 0.021$), but the discrepancy was not as great as on 1% cuticle agar (Figure 6-1b, Table 6-1, see Appendix H).

CuticleSGM*Af6982**Af6982con^{ins}**Af6982**Af6982con^{ins}*

c)

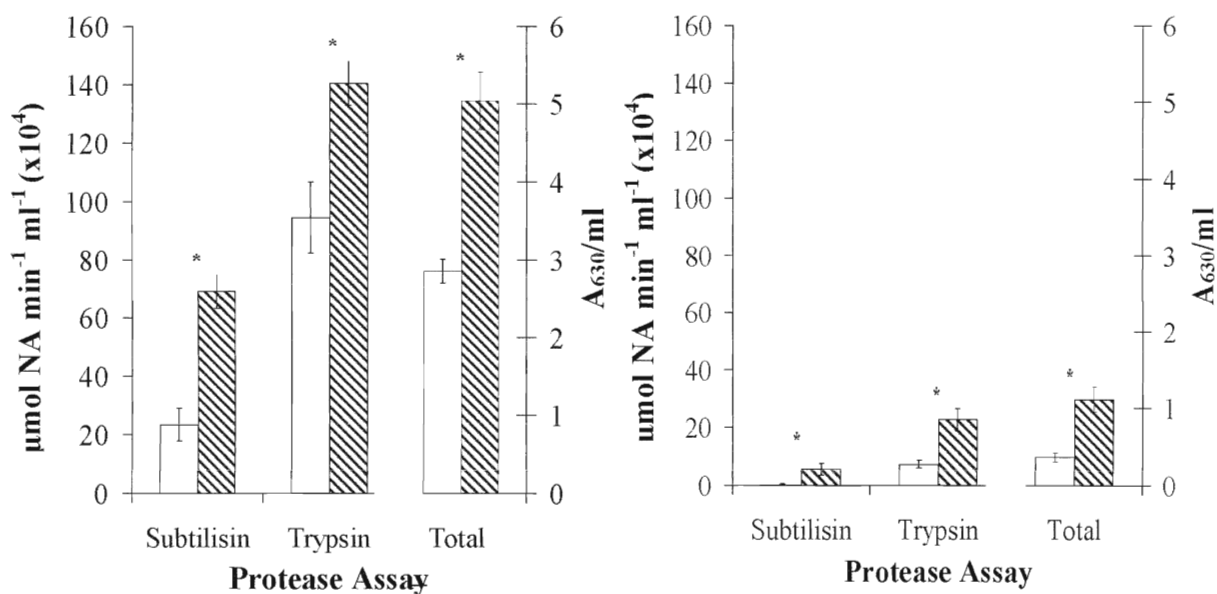


Figure 6-1: Hyphal growth and protease production of *Af6982* and *Af6982con^{ins}* in 1% cuticle and SGM. (a) Colonies grown on agar for 7 d at 30°C. *Af6982con^{ins}* produced only a microscopic amount of growth on 1% cuticle agar (see inset). (b) Microscopic examination of germinating conidia after 18 h or 24 h at 30°C. (c) Subtilisin-like, trypsin-like, or total protease activity present in extracellular filtrate of *Af6982* (white) or *Af6982con^{ins}* (hatched) grown in 1% cuticle liquid media or SGM liquid media for 18 h at 30°C. Data represents three trials.

Table 6-1: GT₅₀ (time to 50% germination) and hyphal growth rate of *Af6982* and *Af6982con*^{ins} during growth on 1% cuticle agar or SGM agar at 30°C. GT₅₀ was calculated using Probit analysis, and a lack of overlap of CI₉₅ was used to determine statistical significance between two values. The hyphal growth rate was calculated as $\Delta \log_{10}$ (average hyphal length (mm))/ Δt (h), and the values represent an average of three independent trials.

Strain	1% Cuticle		SGM	
	GT ₅₀ (CI ₉₅)	Hyphal growth rate \pm S.E. (h ⁻¹)	GT ₅₀ (CI ₉₅)	Hyphal growth rate \pm S.E. (h ⁻¹)
<i>Af6982</i>	7.90 (7.58, 8.23)	0.150 \pm 0.006	6.69 (6.49, 6.90)	0.241 \pm 0.009
<i>Af6982con</i> ^{ins}	21.25 (20.10, 22.46)	0.071 \pm 0.009	6.89 (6.59, 7.20)	0.197 \pm 0.007

Af6982con^{ins} protease production is enhanced in “insect” media

During growth in 1% cuticle liquid media, *Af6982* and *Af6982con^{ins}* secreted proteases, including subtilisin-like and trypsin-like proteases. However, during growth in SGM, protease production by both strains was very low (Figure 6-1c). In both media, *Af6982con^{ins}* exhibited significantly greater amounts of subtilisin-like (cuticle $t = 5.683$, $p < 0.001$; SGM $t = 2.661$, $p = 0.029$), trypsin-like (cuticle $t = 3.187$, $p = 0.007$; SGM $t = 3.761$, $p = 0.004$), and total protease (cuticle $t = 5.464$, $p < 0.001$; SGM $t = 4.368$, $p < 0.001$) activity than *Af6982* (Figure 6-1c). There were no differences in the types of proteases produced by *Af6982* and *Af6982con^{ins}* in cuticle or SGM media when compared using IEF (Figure 6-2). However, *Af6982con^{ins}* showed, qualitatively, more subtilisin-like protease in 1% cuticle than *Af6982*, supporting data in Figure 6-1c).

Af6982con^{ins} requires methionine to downregulate protease production

Af6982 and *Af6982con^{ins}* produced proteases, including subtilisin-like and trypsin-like proteases when grown in media lacking a carbon source (glucose), a nitrogen source (nitrate), or a sulfur source (sulfate). While *Af6982* downregulated protease production when supplied with sulfate in addition to glucose and nitrate (i.e. MM), *Af6982con^{ins}* did not. However, when MM was supplemented with methionine, both *Af6982* and *Af6982con^{ins}* downregulated protease production (Figure 6-3). Furthermore, protease production by *Af6982con^{ins}* in MM without sulfur was similar to protease production in MM, suggesting that in *Af6982con^{ins}* sulfate incorporation was deficient and also resulted in deregulation of protease. IEF revealed no dissimilarities in the types of proteases produced in the presence or absence of glucose, nitrate, or sulfate. Qualitatively, *Af6982con^{ins}* produced more protease than *Af6982* (Figure 6-4).

Af6982 and *Af6982con^{ins}* demonstrate identical adherence and appressoria formation

A. flavus conidia adhered to and germinated on polystyrene Petri dishes in the presence of 0.0125% yeast extract. However, there was no significant difference in percentage adherence of *Af6982* ($16.2\% \pm 4.2$) and *Af6982con^{ins}* ($20.6\% \pm 1.8$) ($\chi^2 = 3.366$; $p = 0.067$). Furthermore,

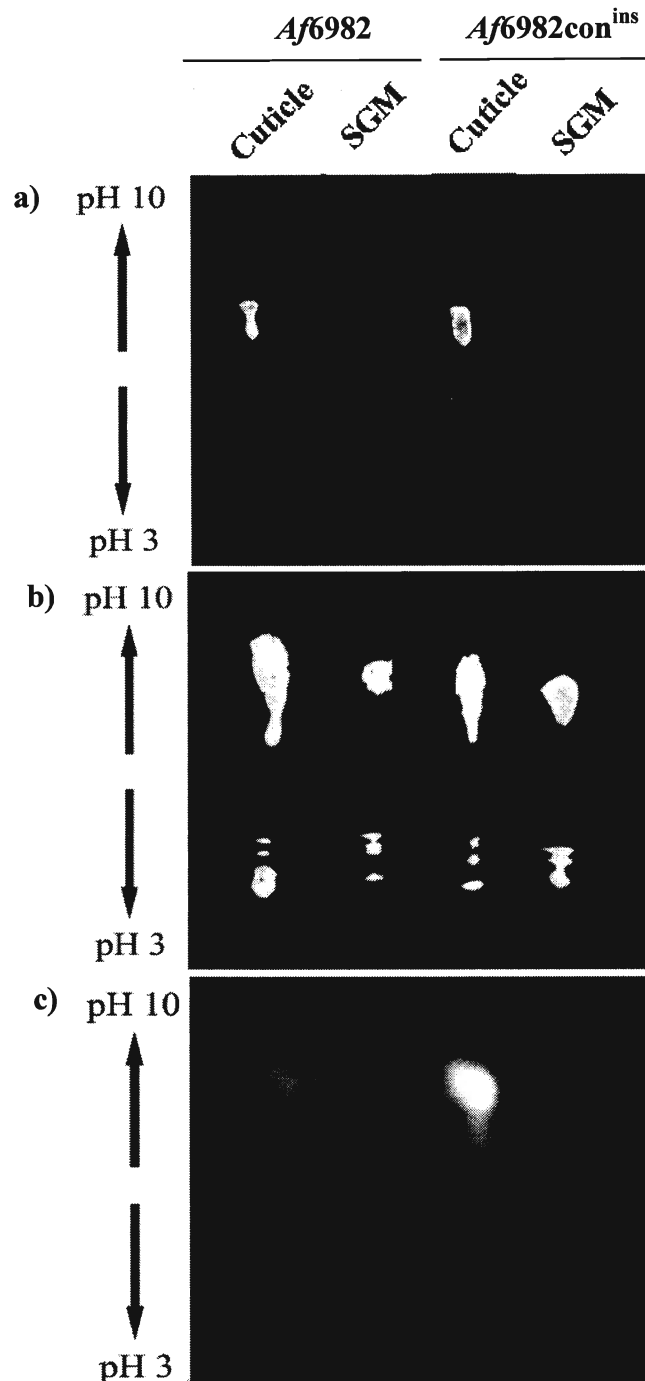


Figure 6-2: Analytical isoelectric focusing (pH 3-10) of extracellular proteases from culture filtrates of *Af6982* and *Af6982con^{ins}* grown in 1% cuticle or SGM at 250rpm at 30°C for 18 h. (a) Gelatin zymography with X-ray film overlay for 30 min at 37°C. (b) Gelatin zymography with X-ray film overlay for 2 h at 37°C. (c) EOM impregnated with subtilisin-like protease substrate succinyl-(alanyl)₂-prolyl-phenylalanine-7-amino-4-trifluoromethylcoumarin with membrane overlay for 3 h at 37°C.

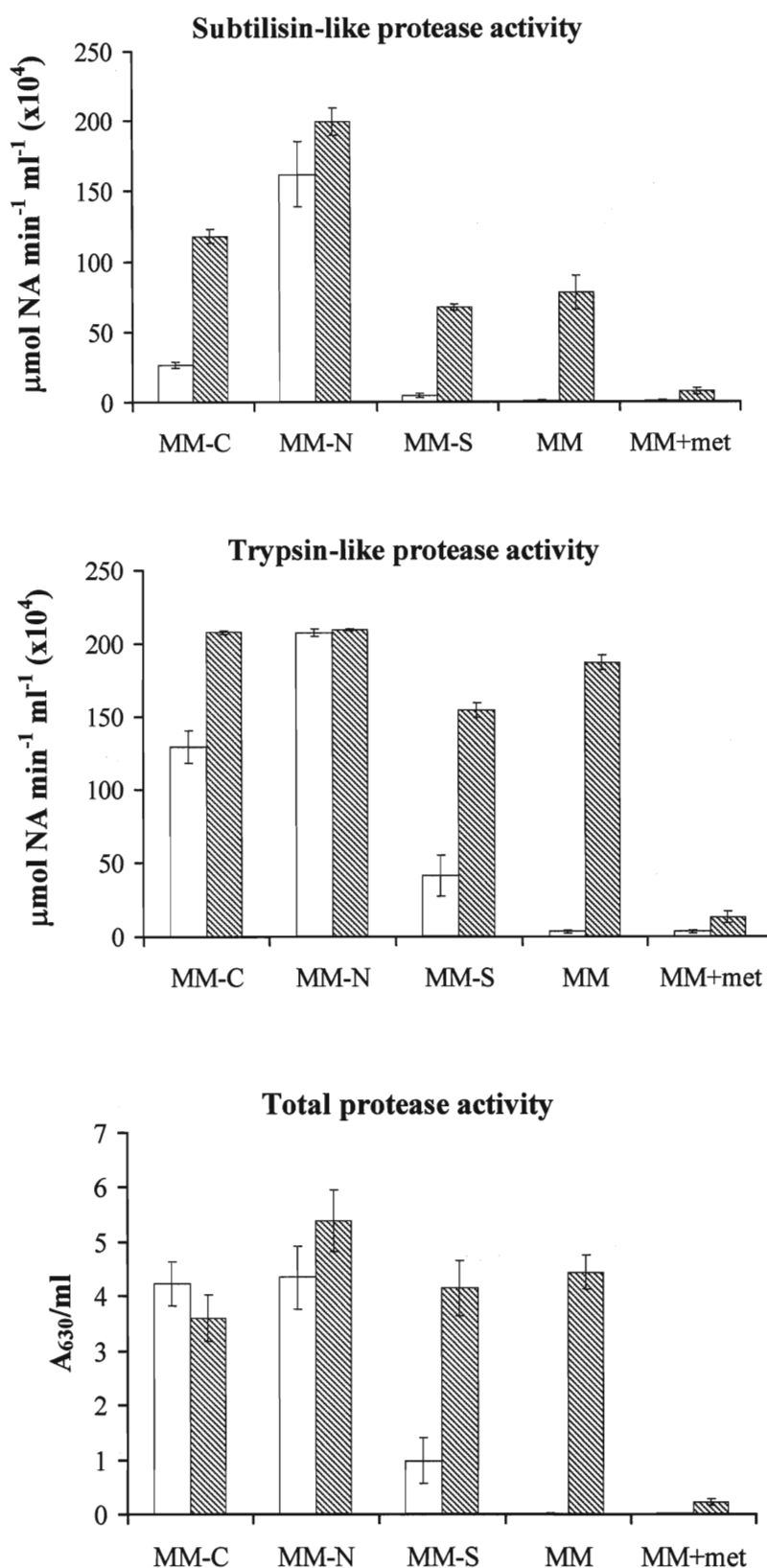


Figure 6-3: Extracellular protease activity from culture filtrates of *Af6982* (white) and *Af6982con^{ins}* (hatched) grown in minimal media (MM), MM lacking carbon (MM-C), MM lacking nitrogen (MM-N), MM lacking sulfur (MM-S), and MM without sulfate but supplemented with 10mM methionine (MM+met) at 250rpm at 30°C for 18 h. Results represent two independent trials.

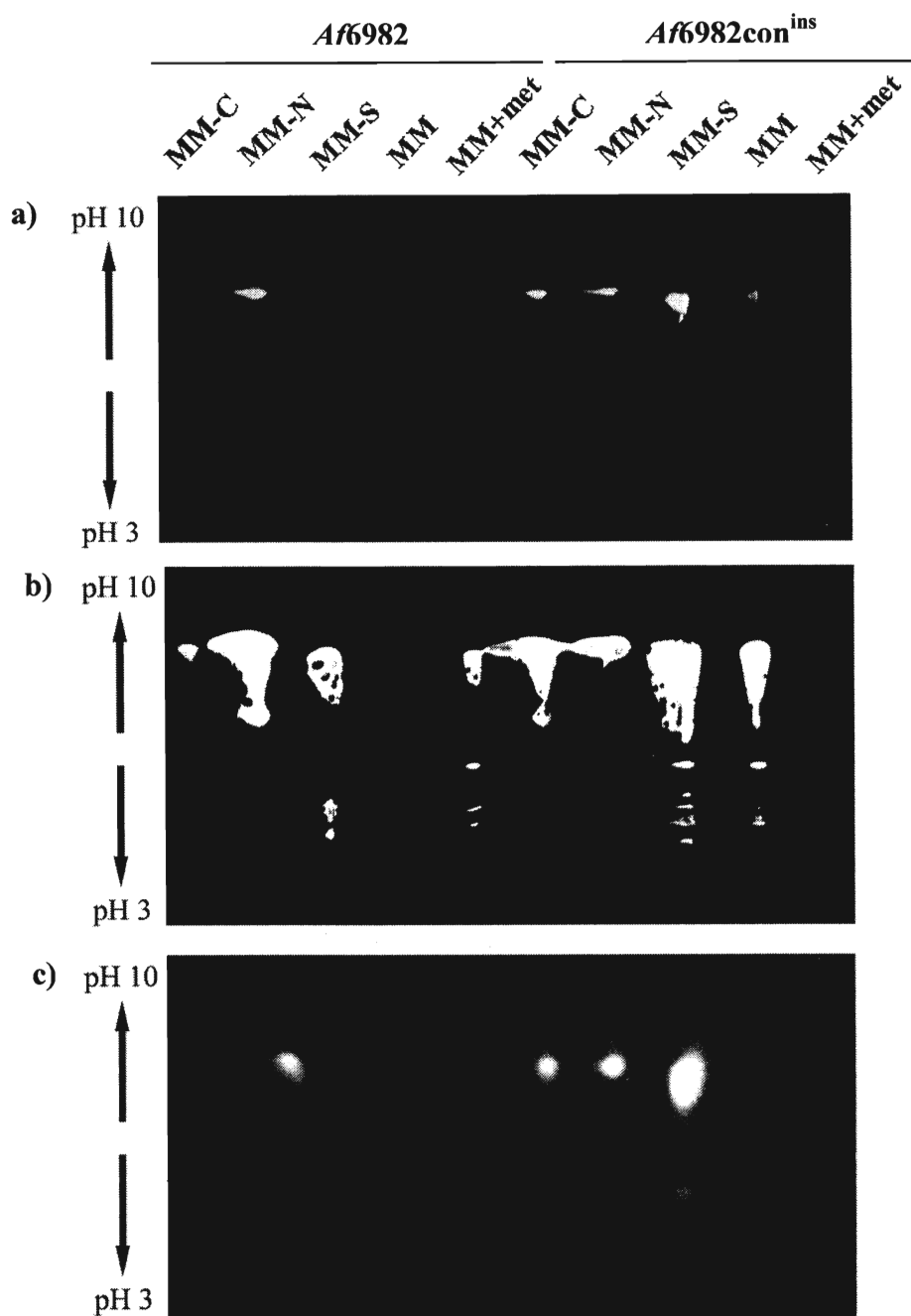


Figure 6-4: Analytical isoelectric focusing (pH 3-10) of extracellular proteases from culture filtrates of *Af6982* and *Af6982con^{ins}* grown in minimal media (MM), MM lacking carbon (MM-C), MM lacking nitrogen (MM-N), MM lacking sulfur (MM-S), and MM lacking sulfate but supplemented with 10mM methionine (MM+met) at 250rpm at 30°C for 18 h. (a) Gelatin zymography with X-ray film overlay for 30 min at 37°C. (b) Gelatin zymography with X-ray film overlay for 2 h at 37°C. (c) EOM impregnated with subtilisin-like protease substrate succinyl-(alanyl)₂-prolyl-phenylalanine-7-amino-4-trifluoromethylcoumarin with membrane overlay for 3 h at 37°C.

there was no significant difference in the percentage of appressoria formed by germinated conidia of *Af6982* ($44.0\% \pm 2.0$) and *Af6982con^{ins}* ($43.4\% \pm 6.4$) on isolated *G. mellonella* larval cuticle (Figure 6-5 a,b) ($\chi^2 = 0.011$; $p = 0.918$). Both strains penetrated the cuticle, and grew through to the interior surface of the cuticle (Figure 6-5 c,d). Cuticle penetration occurred at darkened lesions (Figure 6-5c,d) indicative of a melanization reaction stemming from the prophenyloxidase defense cascade (Kanost et al. 2004).

Af6982 outcompetes *Af6982con^{ins}*

Cz and PDA were used to distinguish *Af6982* and *Af6982con^{ins}* since *Af6982* CFU form on both media, while only PDA supports *Af6892con^{ins}* CFU (Scully & Bidochka 2006a). When various ratios of *Af6982* : *Af6982con^{ins}* conidia were plated on Cz and PDA media, there were no statistically significant differences between $CFU_{CZ} : CFU_{PDA} - CFU_{CZ}$ and the initial ratio plated according to Chi-square tests (10:0, $\chi^2 = 0.090$, $p = 0.764$; 8.75:1.25, $\chi^2 = .044$, $p = 0.830$; 6.25:3.75, $\chi^2 = 2.380$, $p = 0.123$; 3:7, $\chi^2 = 0.460$, $p = 0.498$; 0:10, $\chi^2 = 0$, $p = 1$). Therefore, $CFU_{CZ} : CFU_{PDA} - CFU_{CZ}$ is a good approximation of *Af6982* : *Af6982con^{ins}*.

Sets of 25 *G. mellonella* larvae were infected with various ratios of *Af6982* : *Af6982con^{ins}*. A total of 39 insect cadavers that had been infected with various ratios of *Af6982* : *Af6982con^{ins}* (Table 6-2) were analyzed. Twenty-eight of the cadavers showed no statistically significant difference in $CFU_{CZ} : CFU_{PDA} - CFU_{CZ}$ (t-tests, $p \geq 0.05$), indicating virtually only wild-type conidia. The remaining 11 cadavers exhibited a statistically significant difference in $CFU_{CZ} : CFU_{PDA} - CFU_{CZ}$ (t-tests, $p < 0.05$), indicative of significant amounts of conidia from both strains (Table 6-2). Ten cadavers demonstrated a statistically significant change in the ratio *Af6982* : *Af6982con^{ins}* conidia; 5 had a greater proportion of *Af6982* than initially, while 5 had a greater proportion of *Af6982con^{ins}* than the original inoculum (Table 6-2). Interestingly, one cadaver initially infected with a 1:9 ratio of *Af6982* : *Af6982con^{ins}* produced only *Af6982con^{ins}* conidia (Table 6-2).

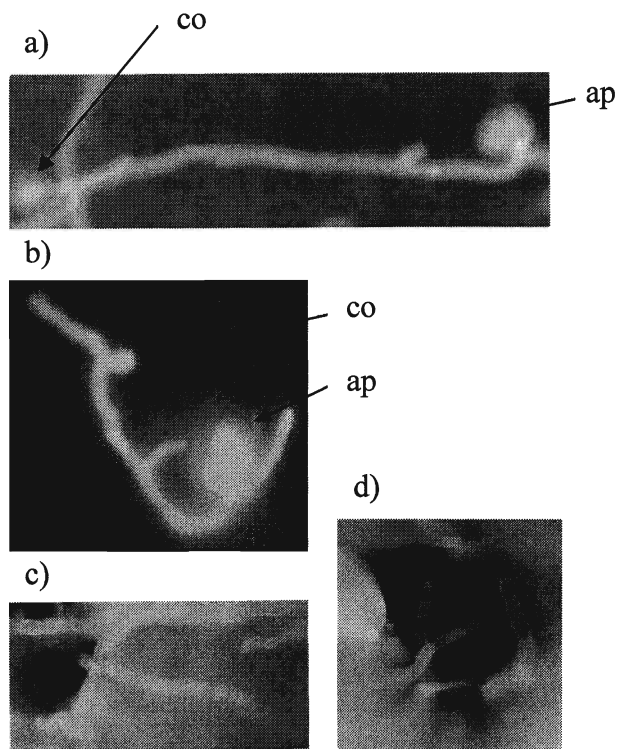


Figure 6-5: Appressoria (ap) differentiated from germinated conidia (co) of (a) *Af6982* and (b) *Af6982con^{ins}* on the surface of *G. mellonella* larval cuticle. Emergent hyphae of (c) *Af6982* and (d) *Af6982con^{ins}* on the interior surface of the cuticle following penetration. Cuticles were incubated for 24 h at 30°C.

Table 6-2: Competition assay between *Af6982* and *Af6982con^{ins}* on *G. mellonella* larvae. Sets of 25 larvae were topically inoculated (5µl of 1x10⁵ conidia ml⁻¹) with various ratios of *Af6982* : *Af6982con^{ins}*. Upon death, insects were surface sterilized and allowed to conidiate for 5 d. The conidia were harvested and plated on 3 Petri dishes each of PDA and Cz. For samples with statistically significant differences in the number of conidia on the PDA and Cz plates (t-test, $\alpha = 0.05$), the final ratio of *Af6982* (CFU_{Cz}) : *Af6982con^{ins}* (CFU_{PDA} – CFU_{Cz}) was determined. Chi-square tests ($\alpha = 0.05$) were employed to determine a statistically significant change in ratio.

Initial ratio (<i>Af6982</i> : <i>Af6982con^{ins}</i>)	No. Insects Examined*	No. Stat. Sig.†	No. CFU _{Cz} : No. CFU _{PDA} - No. CFU _{Cz}	Final Ratio (<i>Af6982</i> : <i>Af6982con^{ins}</i>)
10:0	2	0		
9.4:0.57	5	0		
8.8:1.2	4	2	162:122	5.7:4.3‡
			119:65	6.5:3.5‡
8.1:1.9	4	1	175:47	7.9:2.1
7.3:2.7	4	1	124:107	5.4:4.6‡
6.5:3.5	4	1	153:121	5.6:4.4‡
5.5:4.5	4	0		
4.4:5.6	8	3	109:87	5.6:4.4‡
			90:53	6.3:3.7‡
			126:49	7.2:2.8‡
3.1:6.9	2	1	125:62	6.7:3.3‡
1.7:8.3	4	2	100:107	4.8:5.2‡
			0:272	0:10‡
0:10	4	4	0:313	0:10
			0:228	0:10
			0:195	0:10
			0:235	0:10

*Insect excluded from analysis either had not died 10 days post-infection or failed to produce a substantial amount of conidia.

† Statistically significant difference in number of CFU on PDA and Cz plates (t-test, $\alpha < 0.05$).

‡ Statistically significant change in ratio (Chi-square test, $\alpha < 0.05$).

Discussion

In order to study pathogen evolution and emerging diseases, we employed a model pathogen-host system involving the serial passage of the opportunistic pathogen *A. flavus* through *G. mellonella* larvae (Scully & Bidochka 2005). From this scheme, we isolated a strain of *A. flavus* (*Af6982con^{ins}*) that exhibited traits of an obligate insect pathogen, specifically a drastic reduction in conidial production on PDA and a restricted host range since it continued to infect insects with no alteration in virulence but failed to infect and/or conidiate sufficiently on plants. This phenotype was associated with cysteine/methionine auxotrophy, and the reversion rate suggested a single gene mutation. We hypothesized that an obligate pathogen might evolve when a nutrient deficiency restricts it to a particular host, providing selection pressure for an increase in virulence (Scully & Bidochka 2006a). In this study, we report that the cause of host restriction of *Af6982con^{ins}*, namely cysteine/methionine auxotrophy, has the pleiotrophic effect of increasing virulence factor production (i.e. protease deregulation via sulfur limitation), allowing this slow-growing auxotroph to maintain virulence toward insects.

On 1% cuticle agar and SGM agar, “insect” media intended to mimic respectively either the initial stages of insect pathogenesis when the fungus penetrates the cuticle or the latter stages when the fungus grows extensively in the hemolymph (Clarkson & Charnley 1996), *Af6982con^{ins}* exhibited depressed germination and hyphal growth rates compared to the wild-type. The discrepancy was greatest on 1% cuticle because SGM is nutrient-rich and contains methionine (Dunphy & Webster 1986). The delayed germination and depressed growth of *Af6982con^{ins}* is not unexpected since, by definition, auxotrophs display stunted growth in media containing suboptimal amounts of the required amino acid(s). Since pathogen growth rate is positively correlated with virulence (Brown et al. 2006; Read 1994), *Af6982con^{ins}* should be less virulent than *Af6982*.

However, despite its depressed growth, *Af6982con^{ins}* produced considerably more extracellular subtilisin-like, trypsin-like, and total protease activity than *Af6982* in “insect”

media, although the types of secreted proteases were identical. Incidentally, protease secretion is an important aspect of entomopathogenicity. The facultative fungal insect pathogen *Metarhizium anisopliae* secretes a host of proteases that act as virulence factors affecting cuticle penetration during the initial stages of pathogenesis (St. Leger 1995). Increased protease production in 1% cuticle media indicated that *Af6982con^{ins}* may secrete more virulence-affecting, cuticle-degrading proteases than *Af6982* in vivo during insect pathogenesis. Greater protease secretion by *Af6982con^{ins}* compared to *Af6982* in SGM may also translate to advantages in the insect hemocoel where proteases may aid successful pathogen establishment by cleaving proteins involved in immune system signaling thereby preventing a successful immune response (Griesch & Vilcinskas 1998). Enhanced protease secretion may compensate for the depressed growth of *Af6982con^{ins}*, explaining why it exhibited the same level of virulence toward insects as the faster growing wild-type strain (Scully & Bidochka 2006a).

The induction of extracellular protease production in response to carbon, nitrogen, or sulfur nutrient deficiencies has been reported in *Neurospora crassa*, *Aspergillus nidulans*, and *A. flavus*. Unlike *A. nidulans*, extracellular protease production by *N. crassa* and *A. flavus* has the additional prerequisite of an exogenous protein source (Cohen 1973; Hanson & Marzluf 1975; Srinivasan & Dhar 1990). Furthermore in *N. crassa*, a single protease gene is controlled by three independent regulatory circuits corresponding to the carbon, nitrogen, and sulfur nutrient levels (Hanson & Marzluf 1975). In fungi, the production of extracellular protease in response to carbon starvation is probably mediated by a negative-acting carbon catabolite repressor protein (CRE) (Dowzer & Kelly 1991) since mutations in the *creB* and *creC* genes of *A. nidulans* yield higher levels of extracellular protease (Hynes & Kelly 1977). Positive-acting DNA-binding proteins encoded by *areA* of *A. nidulans* (Kudla et al. 1990) and *nit-2* of *N. crassa* (Fu & Marzluf 1990a; Fu & Marzluf 1990b) mediate protease synthesis in response to nitrogen deprivation as loss-of-function mutations in these genes render the fungus unable to use exogenous protein as a nitrogen source (Arst & Cove 1973; Hanson & Marzluf 1975). Similarly,

N. crassa strains displaying mutations in the *cys-3* gene, which encodes a positive-acting regulatory protein required for the synthesis of enzymes involved in the sulfur assimilation pathway (Kanaan & Marzluf 1993), are incapable of producing protease under sulfur (methionine)-limiting conditions (Hanson & Marzluf 1973).

Likewise, *Af6982* and *Af6982con^{ins}* produced protease under carbon, nitrogen, or sulfur-limiting conditions. In conjunction with glucose and nitrate, either sulfate or methionine repressed protease production by *Af6982*, while only methionine sufficed for *Af6982con^{ins}*. Presumably, this occurred because *Af6982con^{ins}* is a cysteine/methionine auxotroph, incapable of assimilating sulfate as a sulfur source (Scully & Bidochka 2006a). For the same reason, auxotrophy may account for the differential protease secretion in “insect” media.

In entomopathogenic fungi, insect pathogenesis is the outcome of protease secretion in response to starvation. *M. anisopliae* secretes extracellular proteases under carbon and nitrogen deprivation, conditions experienced during germination and growth on the cuticular surface. Upon starvation, protease gene transcription is mediated by CRR1, a carbon catabolite repressor similar to *A. nidulans* CREA, and NRR1, a nitrogen response regulator analogous to AREA from *A. nidulans* (Paterson et al. 1994; St. Leger et al. 1988b; Screen et al. 1997; Screen et al. 1998, St. Leger et al. 1991b). Initially, the secreted proteases degrade cuticular protein (Anderson 1979), releasing nutrients for fungal growth. However, the proteases also act as virulence factors since they ultimately weaken the protective cuticle of the insect, allowing the fungus to infect, colonize, and subsequently kill the insect (St. Leger 1995). Indeed, conidial virulence is correlated concomitantly with both nutrient deprivation (low endogenous CN ratio) and greater amounts of *pr1* transcripts (Shah et al. 2005). Because insect infection by *A. flavus* and *M. anisopliae* is analogous (Clarkson & Charnley 1996; Kumar et al. 2004), this link between starvation, protease production, and insect pathogenesis presumably also occurs in *A. flavus* and suggests that enhanced protease secretion due to auxotrophy has implications regarding the evolution of pathogenesis.

On a biomass basis, *Af6982con^{ins}* secretes significantly more protease than the wild-type during growth on insect cuticle due to its unique requirement for methionine as a sulfur source. Although the depressed germination and hyphal growth rates of *Af6982con^{ins}* should negatively impact entomopathogenesis, enhanced production of virulence-affecting proteases presumably compensates, allowing *Af6982con^{ins}* to maintain virulence toward insects despite auxotrophy. In correlation, there were no differences in virulence, adherence, or appressoria formation of *Af6982* and *Af6982con^{ins}*, factors known to impact entomopathogenesis (Butt et al. 1995). Despite delayed germination, *Af6982con^{ins}* conidia were sufficiently germinated to develop appressoria and penetrate the cuticle at 24 h (Figure 6-1b; Figure 6-5b,d). Given the severely stunted growth of *Af6982con^{ins}* on 1% cuticle after 7 d, *Af6982con^{ins}* is likely unable to obtain adequate supplies of methionine from cuticle alone but must quickly gain access to the nutrient-rich hemolymph (similar to SGM) in order to colonize and kill the insect. Access to the hemocoel may explain the host range limitation of *Af6982con^{ins}* to insects and not plants (Scully & Bidochka 2006a), since plants lack a comparable nutritious cavity. Moreover, enhanced protease secretion would aid insect infection relatively more than plant pathogenesis since protein is the major constituent of insect cuticle, while plant cell walls are primarily composed of carbohydrates. Since both mechanical pressure and proteolytic action cause cuticle penetration (Goettel et al. 1989), *Af6982* may rely more heavily on mechanical pressure afforded by its higher growth rate, while *Af6982con^{ins}* may depend on proteases, yielding alternate pathogenic strategies but indistinguishable cuticular penetration and equal virulence.

Although *Af6982* and *Af6982con^{ins}* were equally virulent, competition assays heavily favored the wild-type as the strain proportionally more abundant on insect cadavers, likely because *Af6982* produced 10 times as many conidia as *Af6982con^{ins}* on insect cadavers (Scully & Bidochka 2006a). Theories on natural selection state that transmission is not necessarily correlated with virulence (Ebert & Herre 1996). For example, when co-infecting insects with *A. flavus* and *M. anisopliae*, *A. flavus* is the more frequent colonizer of the cadavers even though *M.*

anisopliae is the more virulent facultative pathogen (Hughes & Boomsma 2004). Although *Af6982* was more competitive, *Af6982con^{ins}* might be able to survive and evolve in nature, assuming that it successfully locates appropriate niches, which would be dependent on a variety of ecological factors. *Af6982con^{ins}* may also survive as an insect pathogen simply by chance since the pathogen population emerging following host infection is influenced by random, chaotic events in addition to selection (Scully & Bidochka 2006c).

Using a fungal-insect model system, we isolated a strain of *A. flavus* with a mutation conferring cysteine/methionine auxotrophy that accomplished host restriction and increased the level of virulence factor (protease) production, resulting in the adoption of a divergent, more host-specific pathogenic strategy than the opportunistic wild-type strain. Since both changes are notable aspects of pathogen evolution, the results enhance our understanding of this process by highlighting nutrient acquisition as a driving force behind the adaptation of infectious microbes. In correlation, a number of microbial pathogens, including *Salmonella typhimurium*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Campylobacter jejuni*, enterohemorrhagic *Escherichia coli*, *Brucella melitensis*, and *Brucella suis*, harness a starvation response to induce virulence gene expression in reaction to nutritionally limited environments encountered in their hosts in their quest for nutrients (Dozot et al. 2006; Gaynor et al. 2005; Godfrey et al. 2002; Malke et al. 2006; Nakanishi et al. 2006; Pizarro-Cerda & Tedin 2004; Steiner & Malke 2001; Taylor et al. 2002). Moreover, this is one of only a few studies that report an increase in some aspect of virulence due to a genetic or metabolic loss (Maurelli et al. 1998; Maruelli 2007; Moore et al. 2004; Nakata et al. 1993; Parish et al. 2003; Rosqvist et al. 1988; Wren 2003), emphasizing the role of genome decay in the evolution of increased virulence.

Chapter VII:

Experimental genetic transformation of an insect-restricted strain of the opportunistic fungus *Aspergillus flavus* with a protease virulence factor fails to increase pathogen virulence: Lessons in horizontal gene transfer and pathogen evolution

Abstract

Horizontal gene transfer is a major evolutionary pathway in the emergence of novel diseases. Here, its role in enhancing virulence was experimentally tested using a model pathogen-host system involving the opportunistic pathogen *Aspergillus flavus* and *G. mellonella* insect larvae as hosts. An *A. flavus* strain displaying cysteine/methionine auxotrophy and a concomitant host-restriction reminiscent of an obligate insect pathogen was previously derived from a wild-type opportunistic strain (Scully & Bidochka (2006) *Microbiology* 152: 223-232). This strain was transformed with the insect virulence factor protease 1 (Pr1) from the facultative insect pathogen *Metarhizium anisopliae* to investigate the possibility that horizontal gene transfer could further the evolution of a virulent obligate insect pathogen from an opportunistic fungus. Constitutive Pr1 production was achieved but failed to increase virulence toward *G. mellonella* and *T. molitor* insect larvae. Presumably, virulence remained low because protease production was not enhanced but remained 10-fold lower when compared with *M. anisopliae*. Regarding pathogen evolution, this study emphasizes the importance of an appropriate genetic background to accommodate horizontally acquired virulence factors.

Introduction

In addition to the numerous well-known infectious diseases, novel diseases continually emerge, threatening human health and well-being (Taylor et al. 2001). Both pathogen evolution and ecological factors contribute to the emergence of novel diseases. Current theories on pathogen evolution state that facultative and obligate pathogens evolved from opportunistic pathogens (Scheffer 1991) since increased virulence is generally a byproduct of the natural selection of enhanced pathogen fitness (Brown et al. 2006; Read 1994). Opportunistic pathogens in turn evolved from saprobes as the ability to colonize a living host as a new niche offered a selective advantage (Scheffer 1991). Pathogen evolution involves genetic alterations that include horizontal virulence factor acquisition, de novo mutation, and genome decay shaped by the selective pressure imposed by a host. Among these three, horizontal virulence factor acquisition is unparalleled in its potential to dramatically and rapidly affect pathogen evolution (Wren 2003; Brown et al. 2006).

Although emerging diseases are recognized as a significant peril to humans, current research on pathogen evolution primarily involves inferences concerning the evolutionary history of known human pathogens (Stephens & Murray 2001; Waterfield et al. 2004; Wren 2000; Wren 2003). However, a pathogen-host model system subject to experimental manipulation would undoubtedly provide unique insights. To this end, we used a model system involving the fungal pathogen *Aspergillus flavus* and the insect host *Galleria mellonella* larvae. *A. flavus* is an opportunistic pathogen capable in infecting a wide range of hosts, including humans, mammals, insect, and plants, but with low virulence (Yu et al. 2005). Its pathogenic cycle towards insects is reminiscent of other well-characterized entomopathogens (Kumar et al. 2004; Clarkson & Charnley 1996). As an opportunistic pathogen it theoretically possesses the potential of evolving increased virulence and specialization toward a host, indicative of facultative and obligate pathogenesis. Insects are ideal hosts for such a model system because they impart an immune

response similar to the mammalian innate immune response and react to certain pathogens in a manner analogous to mammals (reviewed in Scully & Bidochka 2006b).

Using this fungal pathogen-insect host model system, we serially passed *A. flavus* through *G. mellonella* for five generations (Scully & Bidochka 2005), isolating of a strain of *A. flavus* with characteristics of an obligate insect pathogen. This strain exhibited drastically reduced conidial production or complete lack of growth on agar media and was unable to infect and/or conidiate adequately on plant hosts. However, it maintained pathogenicity toward insects including adequate conidiation as the culmination of its pathogenic cycle. Biochemically, it was a cysteine/methionine auxotroph with an inability to convert sulfate to sulfite as part of the sulfur acquisition pathway (Scully & Bidochka 2006a). We argued that this biosynthetic deficiency conferred characteristics of an obligate insect pathogen since it exhibited a restricted host range and a severe reduction in its ability to grow on artificial media. Furthermore, we proposed that this may be a mechanism by which obligate pathogens evolve since host restriction would theoretically provide the selection pressure for an increase in virulence (Figure 4-6) (Scully & Bidochka 2006a). The purpose of this study was to investigate the latter portion of this hypothetical model by transforming this strain of *A. flavus*, already possessing characteristics of an obligate insect pathogen, with the virulence factor protease 1 (Pr1), known to mediate the essential pathogenic step of cuticle penetration in the facultative insect pathogen *Metarhizium anisopliae* (Clarkson & Charnley 1996), in an attempt to complete the evolution of a virulent obligate insect pathogen from an opportunistic fungus.

Materials and Methods

Strains and culture conditions

Metarhizium anisopliae 2575 (Ma2575) was obtained from the USDA-ARS collection of Entomopathogenic Fungi (Ithaca, New York). *Aspergillus flavus* 6982 (Af6982) was obtained from the University of Alberta Microfungus Collection and Herbarium. *A. flavus* 6982con^{ins} (Af6982con^{ins}) is a phenotypically distinct strain derived from *A. flavus* 6982. It is a

cysteine/methionine auxotroph and exhibits a severe host restriction characterized by insect-restricted conidiation. *Af* 6982con^{ins} displays a drastic reduction in conidial production on agar media and on various plants, but retains pathogenicity towards insects, including adequate conidiation on the insect cadavers (Scully & Bidochka 2006a). All strains were grown on potato dextrose agar (PDA; Difco) at 30°C.

Transformation

Plasmid pPTRI-GFP-pr1A (Figure 7-1) was constructed by inserting expression cassettes for green fluorescent protein (GFP) and protease 1 (Pr1A) of *M. anisopliae* into pPTRI (Takara Bio Inc.) using standard molecular techniques and employing enzymes from New England Biolabs. pPTRI is a chromosomal integrating *Escherichia coli-Aspergillus* shuttle vector containing a pyrithiamine resistance gene from *Aspergillus oryzae* (Kubodera et al. 2002). pTEFEGFP, which contained a red-shifted, mutant GFP cDNA from *Aequorea victoria* controlled by the *Aureobasidium pullulans* TEF promoter and the *Aspergillus awamori gla* terminator (VandenWymelenberg et al. 1997), was digested with *Kpn*I and *Sma*I to remove the GFP expression cassette, which was subsequently ligated into pPTRI at the *Kpn*I and *Sma*I sites to form pPTRI-GFP. pBarGPE1-pr1A, a gift from Carmenza Gongora (Centro Nacional de Investigaciones de Café, Columbia), was digested to remove the *Spe*I/*Nde*I fragment containing the *pr1A* cDNA from *Metarhizium anisopliae* var. *anisopliae* 2575 under the control of the *Aspergillus nidulans gpd* promoter and *trp C* terminator. The *Spe*I/*Nde*I fragment was blunt-end ligated into pPTRI-GFP at the *Sma*I site to create pPTRI-GFP-pr1A.

A. flavus 6982 and *A. flavus* 6982con^{ins} were transformed with pPTRI-GFP-pr1A according to the manufacturers instructions (Takara Bio Inc.). Pyrithiamine-resistant colonies were screened for GFP expression using a Leitz Diaplan microscope with a fluorescein filter set (488nm) (see Appendix I) and for constitutive Pr1 secretion by the degradation of elastin on nutrient agar (Difco) supplemented with 1% GlcNAc, 10mM methionine, and 0.08% insoluble elastin. Constitutive expression of Pr1 resulted in the degradation of elastin in the presence of

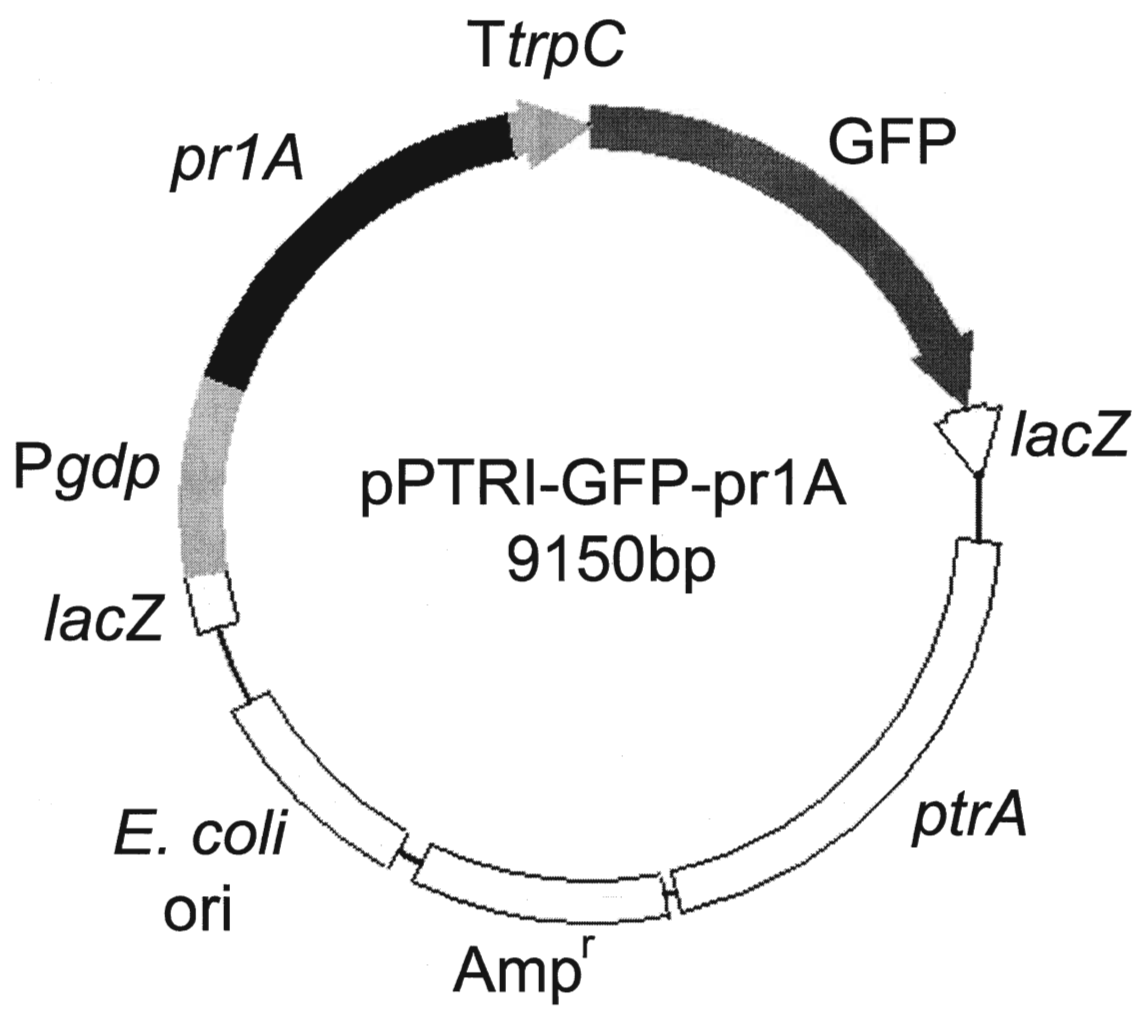


Figure 7-1: pPTRI-GFP-pr1A

GlcNAc and methionine known to repress native protease production (St. Leger et al. 1996). GFP and *pr1*-expressing transformants were purified by single spore isolation and subcultured 5 times on PDA followed by a reconfirmation of GFP and Pr1 expression to verify stable chromosomal integration.

Southern blotting

Genomic DNA, extracted from 3-d-old YPD broth (0.2% yeast extract, 1% peptone, 2% dextrose) cultures as described by Raeder & Broda (1985), was completely digested with *ScaI* and *XhoI* and electrophoresed on a 0.8% agarose gel for 20 h at 23 V at 4°C. Southern blotting to Zeta-Probe nylon blotting membrane (BioRad) and detection using the Roche DIG DNA labeling and detection kit (Roche) was performed according to the manufacturer's protocol. The DIG-labeled probe was constructed from the 1.2 kb *AgeI/XmaI* fragment of pBarGPE1-pr1A, containing a partial sequence of *pr1A* cDNA.

Enzyme Assays

Fungal strains were grown in 100ml YPD broth (0.2% yeast extract, 1% peptone, 2% dextrose) shaking at 250 rpm at 27°C. After 3 d, cultures were filtered from the broth using sterile Büchner funnels, thoroughly washed with sterile dH₂O, and a standard inocula (~2g wet weight) was transferred to either 50ml GlcNAc + met media (1% N-acetylglucosamine, 10mM methionine, 0.02% KH₂PO₄, 0.01% MgSO₄) or 25ml 1% cuticle media. Cuticle from *Locusta migratoria*, courtesy of R. Meldrum Robertson (Queens University, Kingston, Ontario, Canada), was prepared as described by Bidochka and Khachatourians (1990). After 24 h at 250 rpm at 27°C, extracellular filtrates were collected, filtered through 0.2 micron sterile filters, and assayed for subtilisin-like protease activity using the synthetic peptide substrate succinyl-(alanyl)₂-prolyl-phenylalanine *p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-NA) (Sigma) (St. Leger et al. 1987a). The enzyme assays were performed in duplicate and the results were analyzed using ANOVA tests with Tukey's post-hoc comparison (MINITAB).

Isoelectric focusing

Lyophilized extracellular filtrates (see above) were dissolved in 500µl ddH₂O and desalted and concentrated to <50µl by ultrafiltration using Pall Nanosep 10K omega centrifugal devices according to the manufacturer's instructions (Pall). Analytical isoelectric focusing of the samples was performed using Model 111 Mini IEF Cell (BioRad) according to the manufacturer's instructions. Subtilisin-like protease activity was detected by overlaying the IEF gels with cellulose acetate membranes impregnated with the synthetic peptide succinyl-(alanyl)₂-prolyl-phenylalanine-7-amino-4-trifluoromethylcoumarin (MP Biomedicals) (Smith 1984). These enzyme overlay membranes (EOMs) were placed over the gels and incubated for 15 minutes at 37°C. The appearance of fluorescent spots on the EOMs following exposure to UV light was indicative of subtilisin-like protease activity.

Cuticle binding assay

Three-day-old YPD cultures were transferred to 1% cuticle media as described above and grown for 2 d at 27°C and 250 rpm. The extracellular filtrates were collected and desalted by ultrafiltration with Pall Microsep 10K omega centrifugal devices according to the manufacturer's instructions (Pall). The filtrates were diluted to achieve an absorbance of ~0.1 after 15 min at 37°C when assayed for subtilisin-like protease activity against Suc-Ala-Ala-Pro-Phe-NA (St. Leger et al. 1987a). One milliliter of the diluted filtrate was incubated at 4°C with 60mg locust cuticle for 20 min. Following a wash with sterile dH₂O (4°C), the cuticle was incubated at 4°C with 1ml of 0.2M potassium phosphate buffer (pH 7.0) (4°C). Subtilisin-like protease assays were performed on samples from each wash or incubation of the cuticle.

Auxanography

Auxanography was performed as previously described (Scully & Bidochka 2006a).

Bioassays

Galleria mellonella larvae (Peterborough Live Bait, Peterborough, Ontario) and *Tenebrio molitor* larvae (Ward's Scientific) were dipped individually in suspensions (5×10^7 conidia ml^{-1}) prepared in 0.01% Triton X-100 but resuspended in dH_2O (topical application). Alternatively, *G. mellonella* larvae were injected with $3 \mu\text{l}$ of 1×10^5 conidia ml^{-1} . Infected insects were placed individually in plastic snap cap vials (VWR), and incubated at 30°C . Insects were inspected daily to observe death. Upon death, a moist sterile tissue was added to the vial to encourage fungal growth on the cadaver. Three sets of 25 *G. mellonella* larvae and one set of 25 *T. molitor* larvae were topically infected and assessed for percentage mortality by day 10, while one set of 25 *G. mellonella* larvae which were injected were assessed for percentage mortality calculated by day 6. The significance of the percentage mortality was determined using a Chi-square test ($\alpha = 0.05$) with pair-wise post hoc comparisons performed as described by Cox & Key (1993).

Conidial quantification on PDA and *G. mellonella* larvae and pathogenicity testing toward alfalfa (*Medicago sp.*) was performed as previously described (Scully & Bidochka 2006a).

Fluorescence microscopy

G. mellonella larval cuticles were prepared by removing the head and cutting down the ventral midline. The cuticle was separated from the remaining tissue, washed in sterile dH_2O , and inoculated with 5×10^3 conidia. Following 24 h incubation at 30°C , a wet mount of the cuticle was prepared using 0.01% Calcofluor (Fluorescent Brightener 28, Sigma) (Butt 1987). Specimens were viewed and photographed under a Leitz Diaplan microscope with an epifluorescence attachment. Fifty germinated conidia were assessed to determine percentage appressoria formation. Statistically significant differences were determined using Chi-square tests ($\alpha = 0.05$) with pair-wise post hoc comparisons (Cox & Key 1993). The experiments were performed in triplicate.

Results

Construction and characterization of *pr1*-expressing *A. flavus* recombinants

A. flavus strains demonstrating constitutive expression of the *pr1* gene of *M. anisopliae* were constructed by transforming *Af6982* and *Af6982con^{ins}* with the plasmid pPTRI-GFP-pr1A using a protoplast/polyethylene glycol method. pPTRI-GFP-pr1A contains the cDNA of *pr1A* from *M. anisopliae* under the control of the *A. nidulans gpd* constitutive promoter in addition to the GFP cDNA from *A. victoria* controlled by the *A. pullulans TEF* promoter and a pyrithiamine resistance gene from *A. oryzae*. Putative transformants, initially selected based on resistance to pyrithiamine and the presence of GFP when viewed with fluorescence microscopy (see Appendix I), were further screened for constitutive protease expression as evidenced by the degradation of elastin under nutrient-rich conditions that suppress native *A. flavus* protease production. Three recombinants each of *Af6982* and *Af6982con^{ins}* demonstrating constitutive protease expression were purified by single spore isolation and once more tested for pyrithiamine resistance, GFP production, and constitutive protease expression. These recombinants were morphologically indistinguishable from the non-recombinants and maintained both GFP production and constitutive protease expression following five subcultures on PDA. These recombinants were designated *Af6982* pPTRI-GFP-pr1A #1-3, and *Af6982con^{ins}* pPTRI-GFP-pr1A #1-3.

Furthermore, *A. flavus* strains *Af6982* and *Af6982con^{ins}* were transformed with the plasmid pPTRI-GFP to obtain recombinants that were pyrithiamine resistant and expressed GFP but did not contain *pr1A*. One recombinant each of *Af6982* and *Af6982con^{ins}* demonstrating GFP production was purified by single spore isolation and tested for pyrithiamine resistance and GFP production. Morphological and phenotypic stability with respect to GFP production and pyrithiamine resistance was confirmed following five subcultures on PDA. These recombinants, designated *Af6982* pPTRI-GFP and *Af6982con^{ins}* pPTRI-GFP, failed to degrade elastin under nutrient rich conditions.

Auxanographic testing showed that *Af6982con^{ins}* pPTRI-GFP-pr1A #1-3 and *Af6982con^{ins}* pPTRI-GFP displayed the cysteine/methionine auxotrophic phenotype characteristic of *Af6982con^{ins}* (Figure 7-2).

Southern blotting using a DIG-labelled fragment from pBarGPE1-pr1A containing a partial sequence of the cDNA of *pr1A* from *M. anisopliae* confirmed the presence of *pr1A* in the genome of the recombinants (Figure 7-3). Either one or two copies of *pr1A* were present in the transformants. Restriction patterns for *Af6982* pPTRI-GFP-pr1A #2, *Af6982con^{ins}* pPTRI-GFP-pr1A #1, *Af6982con^{ins}* pPTRI-GFP-pr1A #2, and *Af6982con^{ins}* pPTRI-GFP-pr1A #3 were similar, suggesting similar integration sites for *Pr1*.

Constitutive protease expression in *A. flavus pr1* recombinants

M. anisopliae 2575 (*Ma2575*) and the recombinant and non-recombinant strains of *A. flavus* were assessed for subtilisin-like protease production characteristic of Pr1 under nutrient-rich conditions where *M. anisopliae* protease production is down-regulated (Figure 7-4a). Subtilisin-like protease activity was present in the extracellular filtrate of the *A. flavus pr1A* recombinants grown in 1% GlcNAc + 10mM met media, while *Ma2575*, *Af6982*, *Af6982con^{ins}*, *Af6982* pPTRI-GFP, and *Af6982con^{ins}* pPTRI-GFP produced only minor amounts of subtilisin-like protease activity in this nutrient-rich media known to suppress protease synthesis under native control (Figure 7-4a). During growth in 1% cuticle media, statistically similar levels of subtilisin-like protease activity were produced by all of the *A. flavus* strains, however, *Ma2575* produced approximately 10 times more subtilisin-like protease than any of the *A. flavus* strains (Figure 7-4b) ($F = 255.28$; $p < 0.001$; Tukey post-hoc comparison).

The proteins secreted by the *M. anisopliae* and *A. flavus* strains grown in 1% GlcNAc + 10mM methionine or 1% cuticle media were separated using analytical isoelectric focusing and subtilisin-like protease activity was detected using an enzyme overlay membrane. During growth in 1% GlcNAc + 10mM methionine, only the *A. flavus pr1A* transformants produced subtilisin-like protease, which demonstrated a highly basic pI characteristic of *M. anisopliae* Pr1

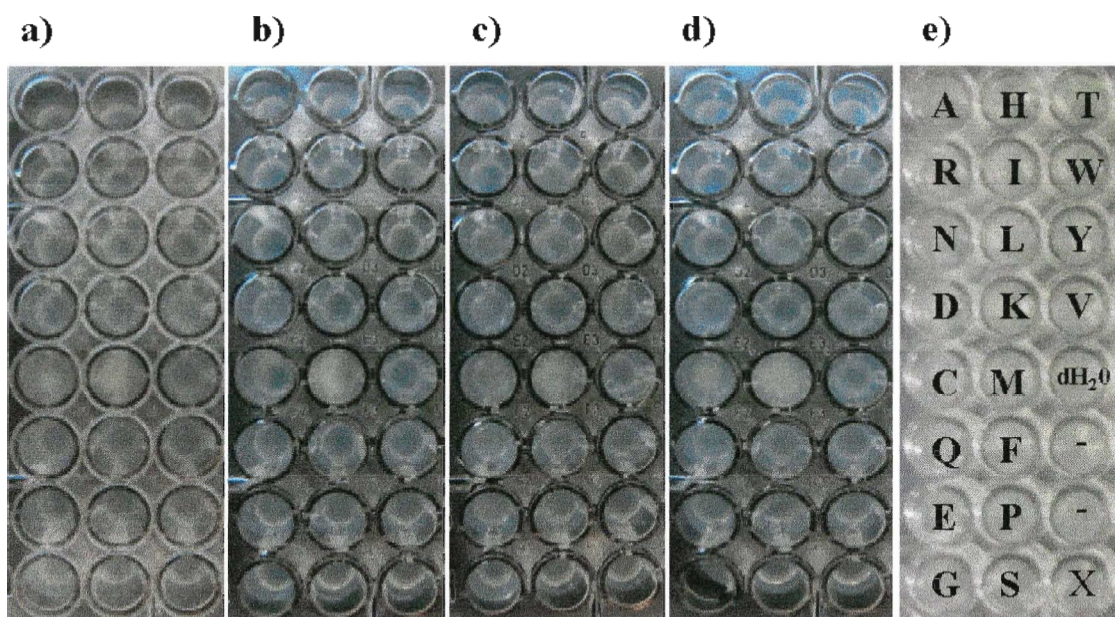


Figure 7-2: Growth of (a) *Af6982con^{ins}* pPTRI-GFP-pr1A#1 (b) *Af6982con^{ins}* pPTRI-GFP-pr1A#2 (c) *Af6982con^{ins}* pPTRI-GFP-pr1A#3 and (d) *Af6982con^{ins}* pPTRI-GFP on Cz media supplemented with various amino acids. Wells containing 200μl of Cz agar were supplemented with 10mM of the amino acid indicated in the key (e). Wells designated “-” were not supplemented. All wells except “X” were inoculated with 5μl of a 1×10^6 conidia ml⁻¹ and incubated at 30°C for 3 d.

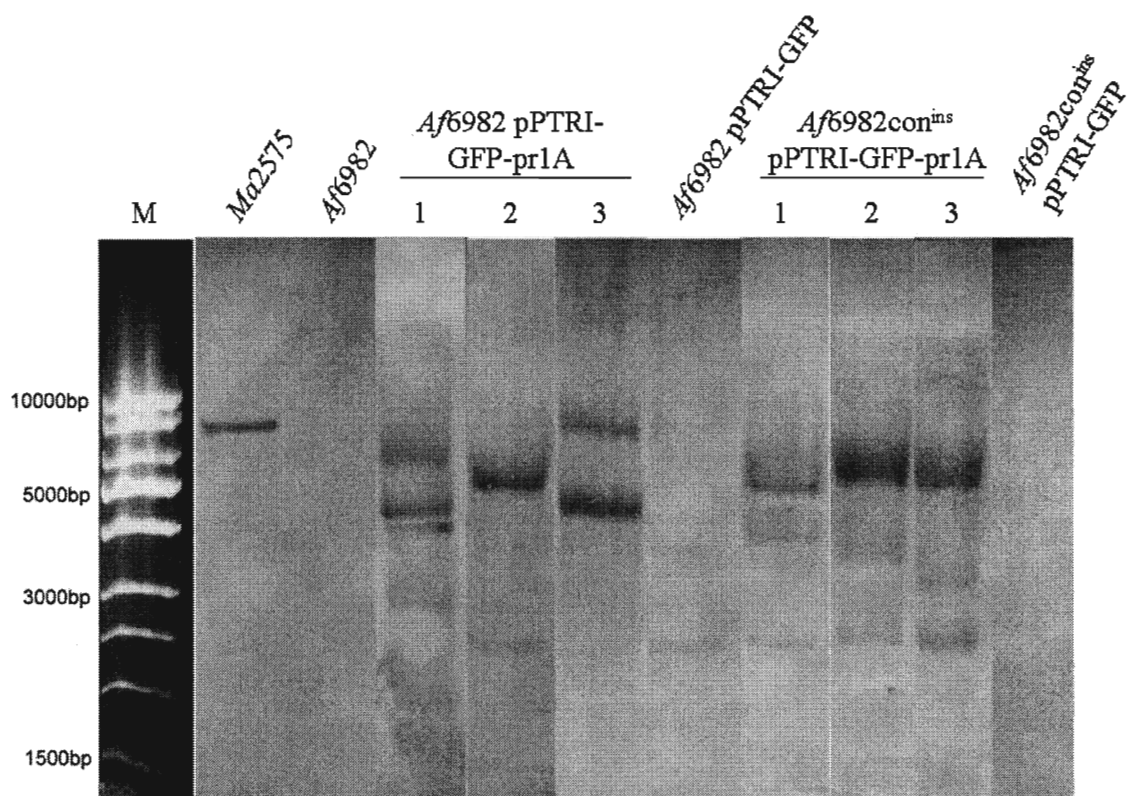


Figure 7-3: Southern blot analysis of total genomic DNA digested with *ScaI* and *XhoI* and probed with a digoxigenin-labeled 1.2-kb *XmaI/AgeI* fragment of pBarGPE1-pr1A containing the cDNA of *pr1A* of *M. anisopliae*. (M) molecular weight marker.

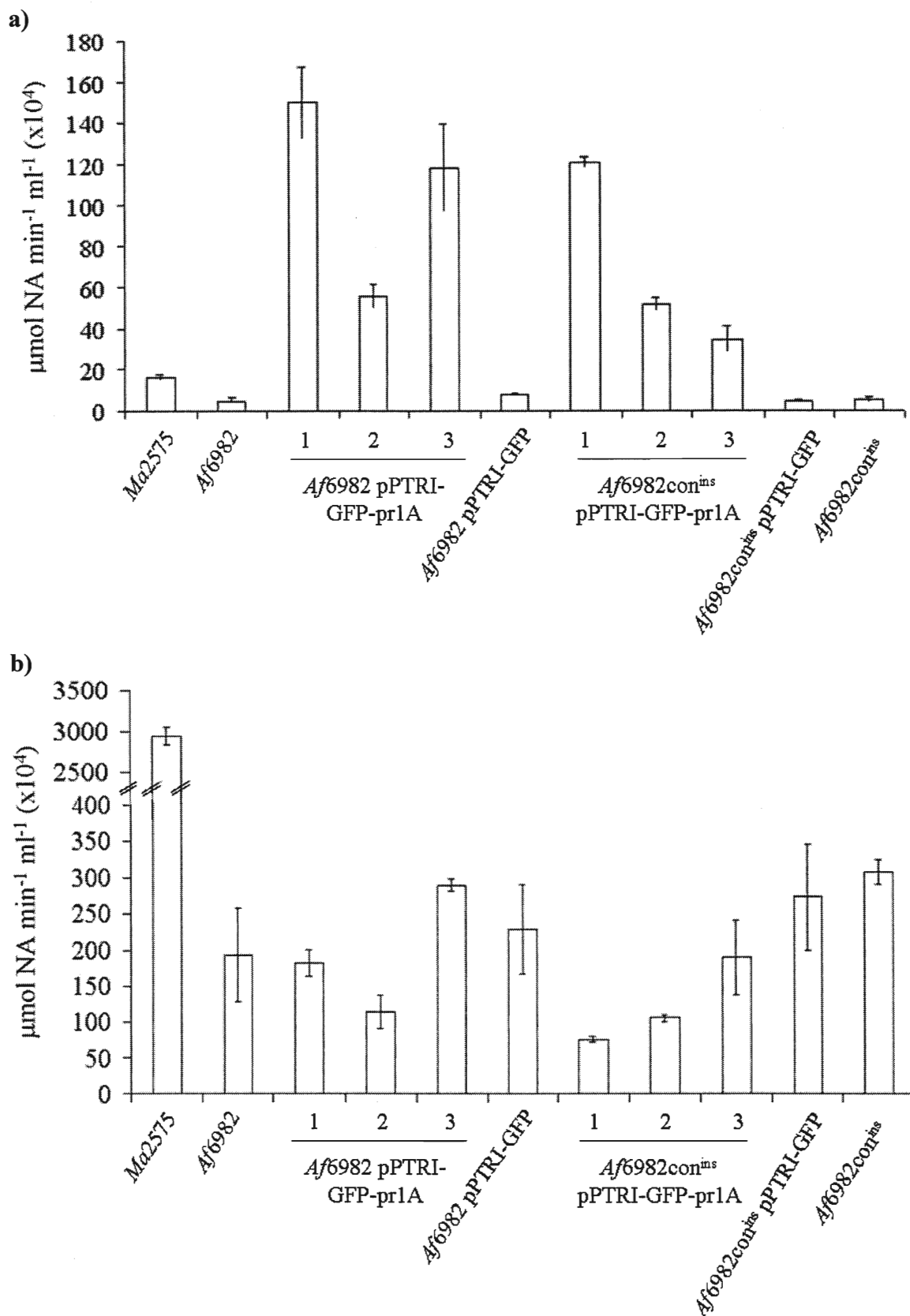


Figure 7-4: Subtilisin-like protease activity in culture filtrates of *M. anisopliae* and *A. flavus* strains grown in (a) 1% GlcNAc + 10mM methionine or (b) 1% cuticle. Samples were assayed against the substrate Suc-ala-ala-pro-phe-NA, and the amount of nitroanalide released was determined from A₄₁₀.

(St. Leger et al. 1987a) (Figure 7-5a). In 1% cuticle media, all of the *A. flavus* strains produced subtilisin-like proteases, the most abundant of which had pI ~ 8. However, the *pr1A* recombinants also secreted a protease with the same highly basic pI (~10) as the very prominent subtilisin-like protease produced by *M. anisopliae* in 1% cuticle media (Figure 7-5b).

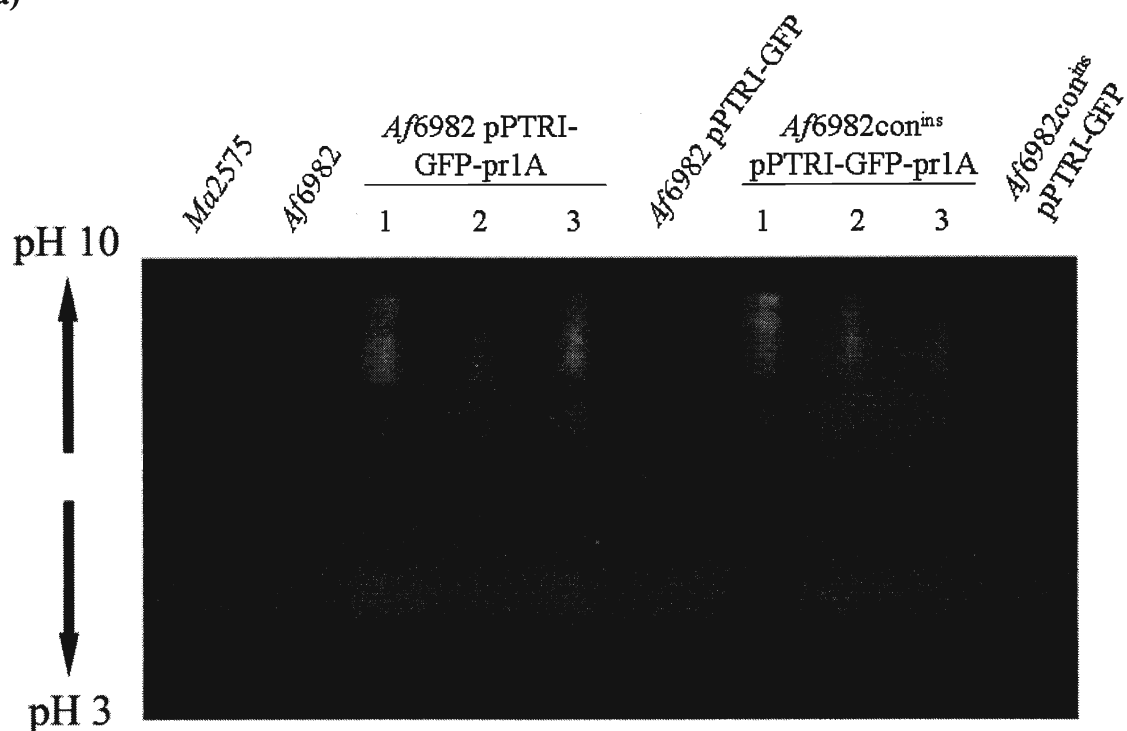
Binding efficiency of proteases of *M. anisopliae* and *A. flavus* to insect cuticle

The binding efficiency of the *M. anisopliae* and *A. flavus* subtilisin-like proteases to insect cuticle was assessed by incubating cuticle with extracellular filtrates collected from cultures grown in 1% cuticle. Unlike either *Af6982* or *Af6982con^{ins}*, where 75.3% and 70.9% respectively of the proteases remained free in the supernatant, only 34.0% of *Ma2575* protease was found in the supernatant following incubation with insect cuticle (Figure 7-6a,b,c; column ii). While similar percentages of the original amount of subtilisin-like protease activity were present in the water (9.0% and 4.3% respectively) and potassium phosphate buffer (22.0% and 17.8% respectively) washes of the *Af6982* and *Af6982con^{ins}* samples (Figure 7-6b,c; column iii), only 4.1% of the original amount of the *Ma2575* protease activity was recovered in the water wash, while 48.4% was reclaimed with the potassium phosphate buffer wash (Figure 7-6a, column iii). The recombinants *Af6982* pPTRI-GFP-pr1A #3 and *Af6982con^{ins}* pPTRI-GFP-pr1A #2 demonstrated binding properties intermediate of *A. flavus* and *M. anisopliae* (Figure 7-6d,e).

Pathogenicity and virulence toward *G. mellonella* and alfalfa

Both topical infection and injection of *G. mellonella* larvae with *M. anisopliae* and *A. flavus* strains resulted in statistically significant percentage mortality compared to a control inoculation with dH₂O according to a Chi-square tests (topical $\chi^2 = 102.87$, df = 11, $p < 0.001$; injection $\chi^2 = 64.83$, df = 11, $p < 0.001$) with post hoc pair-wise comparisons (Cox & Key 1993) (Table 7-1). *Ma2575* inflicted significantly greater percentage mortality than the *A. flavus* strains with both topical infection and injection. The *pr1*-expressing and non-*pr1*-expressing *A. flavus* strains demonstrated no significant differences in percentage mortality of *G. mellonella* larvae except for the topical application of *Af6982* pPTRI-GFP-pr1A #1 and *Af6982con^{ins}*

a)



b)

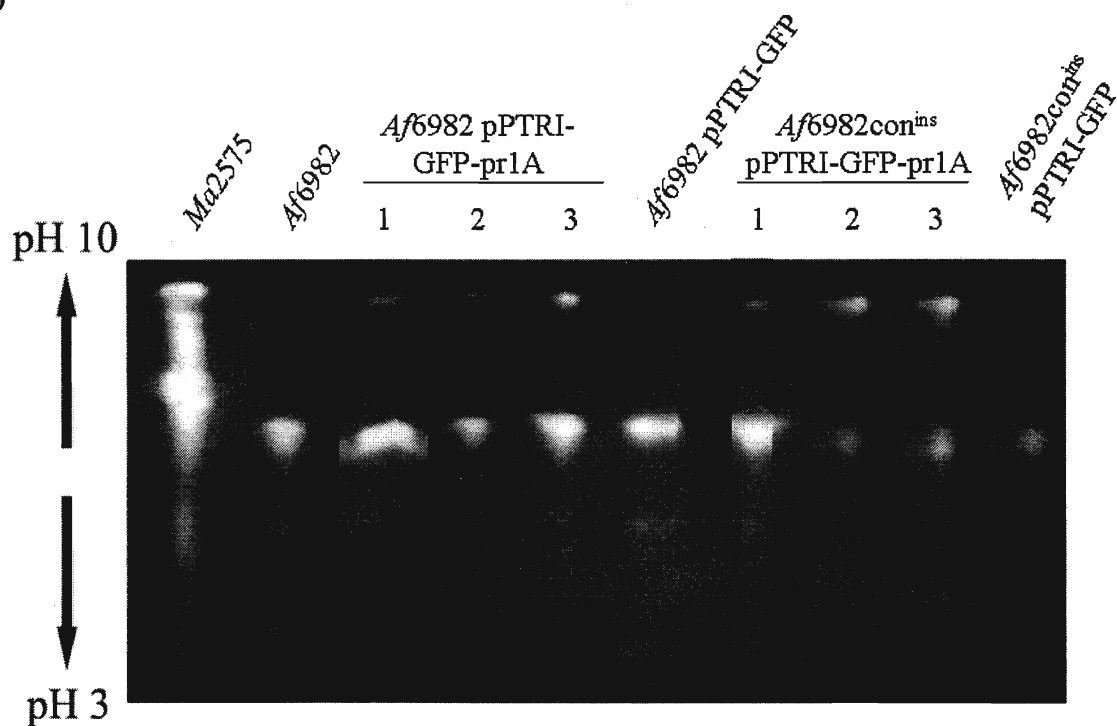


Figure 7-5: Analytical isoelectric focusing (pH 3-10) and enzyme overlay membrane (Suc-ala-ala-pro-phe-AFC) detection of subtilisin-like proteases found in culture filtrates of *M. anisopliae* and *A. flavus* strains grown in (a) 1% GlcNAc + 10mM methionine or (b) 1% cuticle.

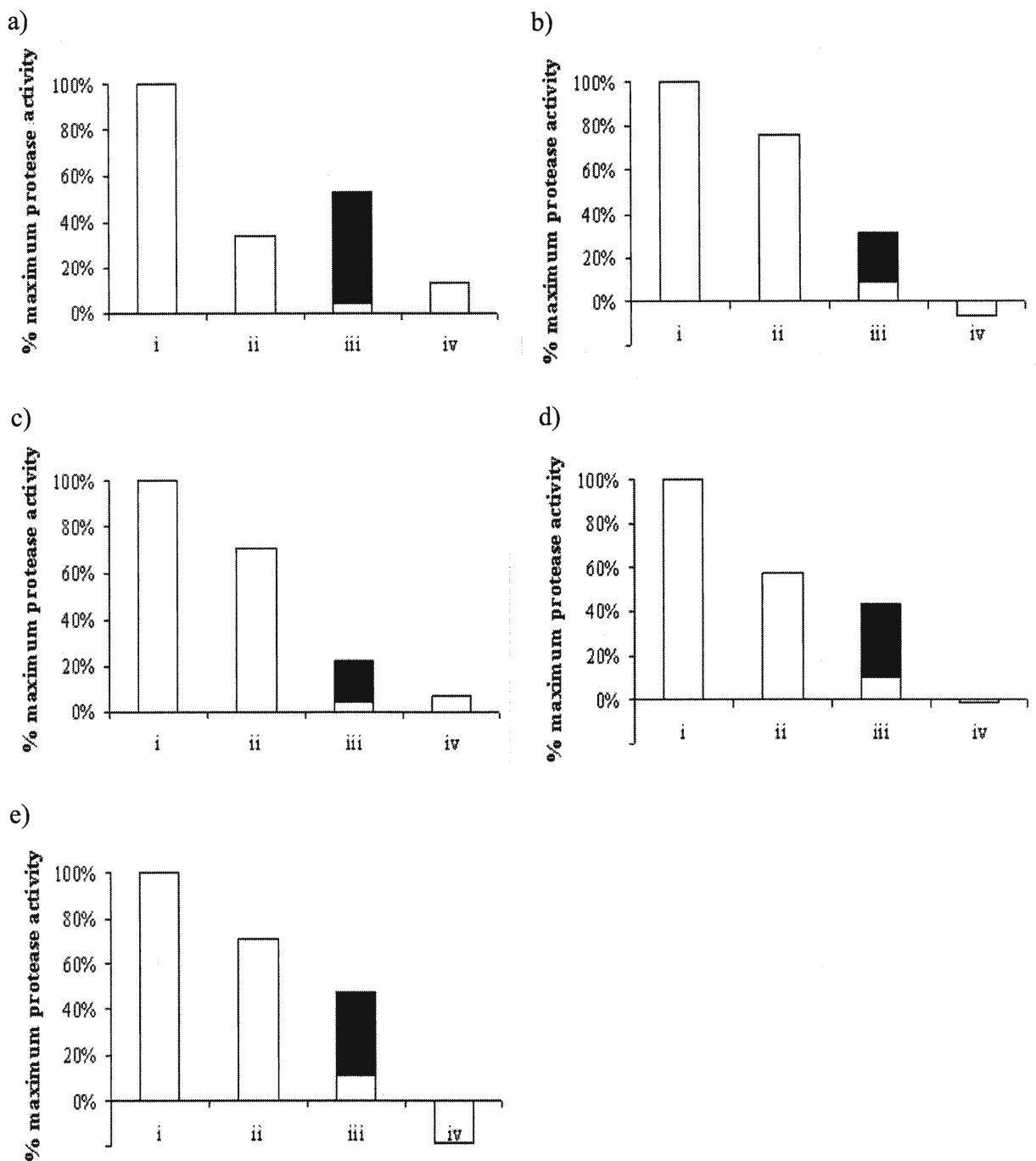


Figure 7-6: Insect cuticle binding assay of subtilisin-like proteases secreted by *M. anisopliae* and *A. flavus* strains during growth in 1% cuticle media. (a) *Ma2575* (b) *Af6982* (c) *Af6982con^{ins}* (d) *Af6982* pPTRI-GFP-pr1A 3# (e) *Af6982con^{ins}* pPTRI-GFP-pr1A #2. One milliliter of filtrate was incubated for 20 minutes at 4°C with 60 mg cuticle, and the percentage of subtilisin-like protease activity remaining in the filtrate (ii) was assessed relative to a control sample of the filtrate (i). Subsequently, the amount of subtilisin-like protease activity present in a ddH₂O wash of the cuticle (iii bottom) and following incubation with 0.2M potassium phosphate buffer pH 7.0 for 30 min at 4°C (iii top) was determined. Columns ii and iii were subtracted from column i to calculate any excess or unaccounted for protease (iv).

Table 7-1: Virulence of *A. flavus* and *M. anisopliae* strains towards *G. mellonella* assessed as the percentage mortality by day 10 after topical application by dipping 3 sets of 25 insects in 5×10^7 conidia ml^{-1} suspensions or by day 6 following injection of 1 set of 25 insects with 5 μl of 1×10^5 conidia ml^{-1} , virulence towards *T. molitor* assessed as percentage mortality by day 10 after topical application by dipping 1 set of 25 larvae in 5×10^7 conidia ml^{-1} suspensions, and conidiation properties of *A. flavus* and *M. anisopliae* strains on alfalfa (*Medicago sp.*) (+ conidiation; - no conidiation).

Strain	Mortality (%) towards <i>G. mellonella</i>		Mortality (%) towards <i>T.</i> <i>molitor</i>	Conidiation on Alfalfa
	Top. appl.	Injection	Top. appl.	
Control (dH ₂ O)	16% \pm 6	4%	8%	-
<i>Ma2575</i>	85% \pm 8	100%	76%	-
<i>Af6982</i>	31% \pm 9	44%	8%	+
<i>Af6982</i> pPTRI-GFP-pr1A #1	25% \pm 7	28%	24%	+
<i>Af6982</i> pPTRI-GFP-pr1A #2	36% \pm 11	24%	12%	+
<i>Af6982</i> pPTRI-GFP-pr1A #3	35% \pm 7	28%	8%	+
<i>Af6982</i> pPTRI-GFP	45% \pm 15	20%	4%	+
<i>Af6982con</i> ^{ins} pPTRI-GFP-pr1A #1	39% \pm 15	28%	12%	-
<i>Af6982con</i> ^{ins} pPTRI-GFP-pr1A #2	25% \pm 9	36%	8%	-
<i>Af6982con</i> ^{ins} pPTRI-GFP-pr1A #3	41% \pm 9	48%	12%	-
<i>Af6982con</i> ^{ins} pPTRI-GFP	33% \pm 7	32%	8%	-
<i>Af6982con</i> ^{ins}	35% \pm 9	44%	24%	-

pPTRI-GFP-pr1A #2, which demonstrated significantly lower percentage mortalities than topical infection with *Af6982con^{ins}*, *Af6982* pPTRI-GFP-pr1A #2, *Af6982* pPTRI-GFP-pr1A #3, *Af6982* pPTRI-GFP, *Af6982con^{ins}* pPTRI-GFP-pr1A #1, *Af6982con^{ins}* pPTRI-GFP-pr1A #3, and *Af6982con^{ins}* pPTRI-GFP, and injection with *Af6982con^{ins}* pPTRI-GFP-pr1A #3, which demonstrated a significantly higher percentage mortality than injection with *Af6982* pPTRI-GFP (Table 7-1). While *Ma2575* inflicted a statistically significant percentage mortality toward *T. molitor* larvae, the transformed and non-transformed strains of *Af6982* and *Af6982con^{ins}* failed to do so ($\chi^2 = 74.911$, $df = 11$, $p < 0.001$) (Cox & Key 1993) (Table 7-1). Unlike *Af6982* strains, all *Af6982con^{ins}* strains failed to conidiate on alfalfa leaves (Table 7-1).

As previously reported, *Af6982con^{ins}* characteristically produces 10 fold fewer conidia on *G. mellonella* insect cadavers and 100 fold fewer conidia on PDA compared with *Af6982* (Scully & Bidochka 2006a). *Af6982con^{ins}* pPTRI-GFP-pr1A #1-3, and *Af6982con^{ins}* pPTRI-GFP also produced approximately 10 fold fewer conidia on *G. mellonella* insect cadavers and 100 fold fewer conidia on PDA compared with any of the *Af6982* strains (Figure 7-7).

Insect cuticle penetration

Both *M. anisopliae* and *A. flavus* produced appressoria on the isolated cuticle of *G. mellonella* larvae and penetrated the cuticle (Figure 7-8). Appressoria and penetrant hyphae were found at darkened areas of the cuticle, evidence of a melanization reaction resulting from the insect prophenyloxidase defense system (Kanost et al. 2004). A significantly greater percentage of germinated *M. anisopliae* conidia produced appressoria ($68.0\% \pm 2.3$) than any of the *A. flavus* strains ($42.7 - 44.7\%$) ($\chi^2 = 30.04$, $df = 4$, $p < 0.001$) according to post-hoc comparisons (Cox & Key 1993). There was no significant difference in the percentage of germinated conidia that formed appressoria among the *pr1*-expressing ($42.7\% \pm 6.8$ for *Af6982* pPTRI-GFP-pr1A #3; $44.7\% \pm 4.7$ for *Af6982con^{ins}* pPTRI-GFP-pr1A #2) and non-*pr1*-expressing ($44.0\% \pm 2.0$ for *Af6982*; $43.4\% \pm 6.2$ for *Af6982con^{ins}*) *A. flavus* strains.

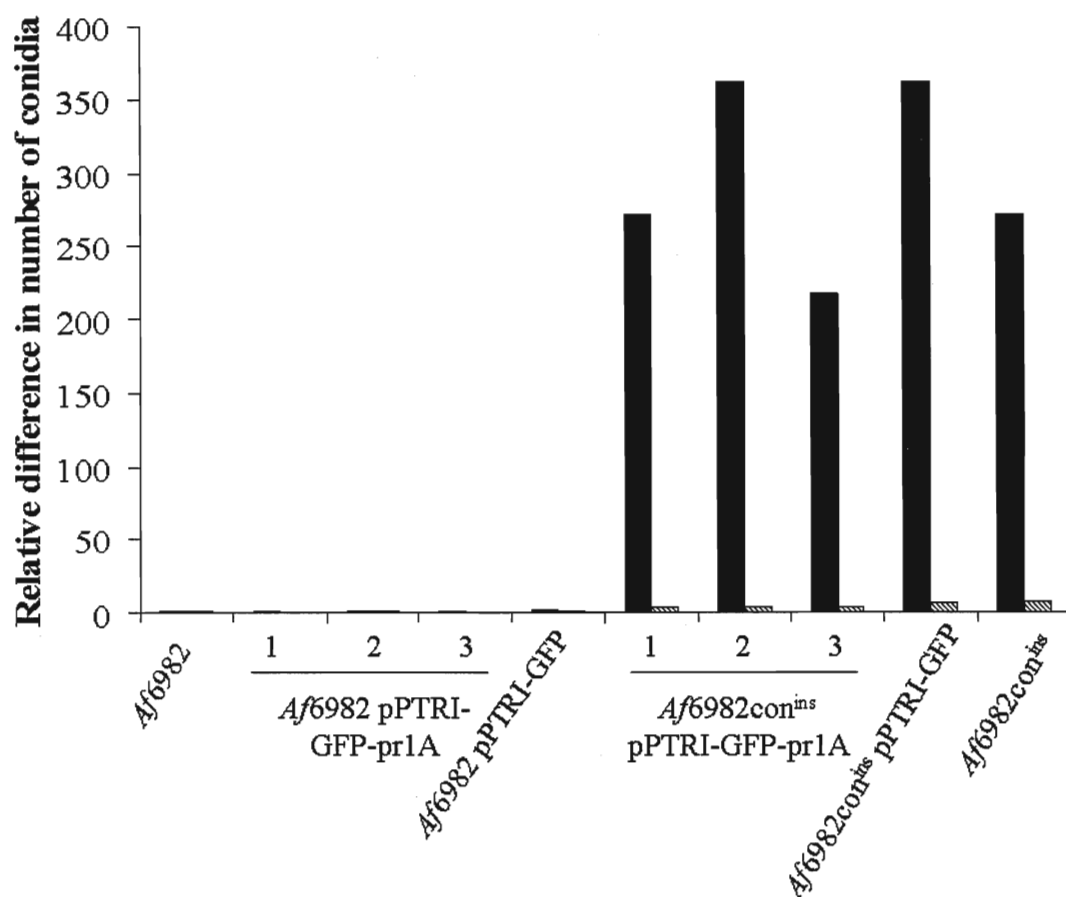


Figure 7-7: Relative difference in number of conidia produced by *Af6982* and various transformed and non-transformed strains of *Af6982* and *Af6982con^{ins}* grown on PDA (black bars) and *G. mellonella* larvae (hatched bars). The relative difference was calculated as the number of conidia produced by *Af6982* divided by the number of conidia produced by the indicated transformed or non-transformed strain of *Af6982* or *Af6982con^{ins}*. The amount of conidia on *G. mellonella* larvae was assessed after topical application (dip in 5×10^7 conidia ml⁻¹). The amount of conidia was calculated as the mean of three replicates.

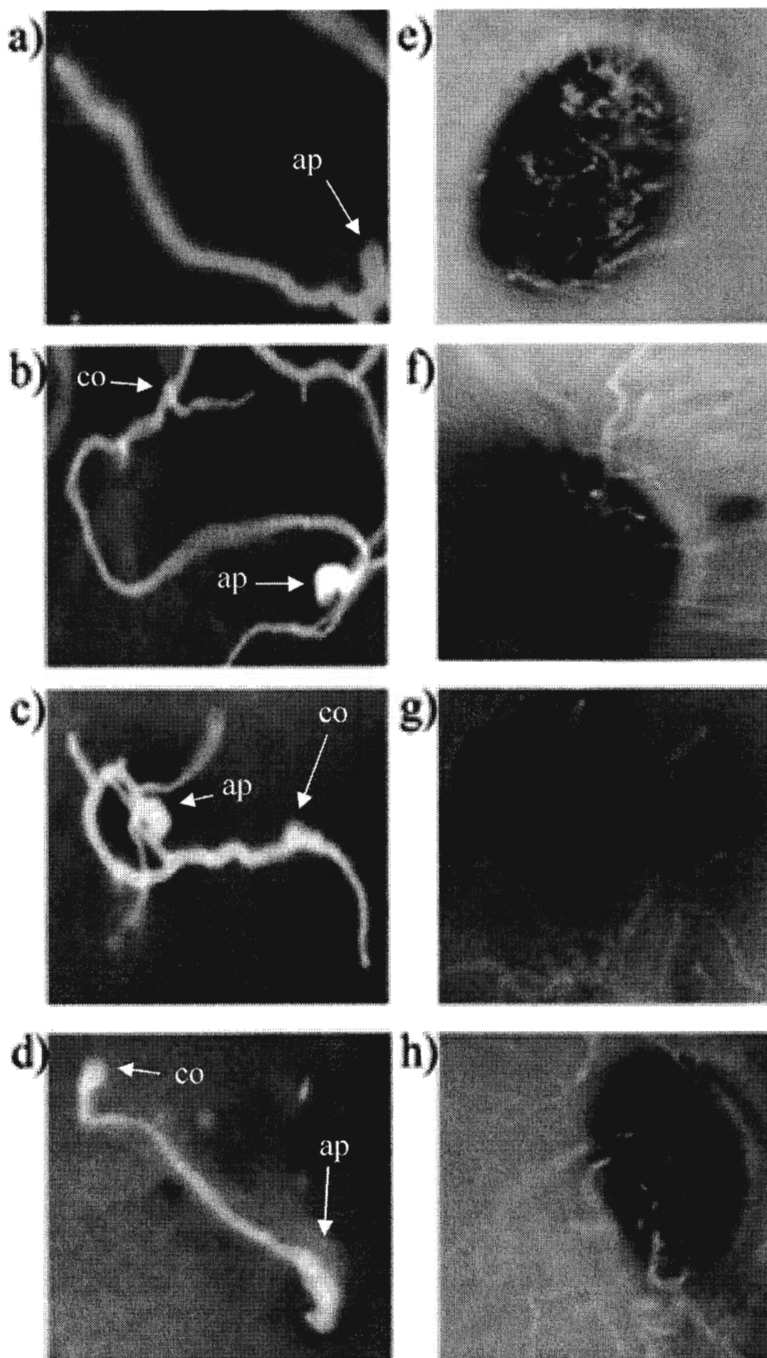


Figure 7-8: Formation of appressoria (ap) on isolated *G. mellonella* larval cuticle by germinated conidia (co) of (a) Ma2575 (b) *Af6982* (c) *Af6982con*^{ins} (d) *Af6982con*^{ins} pPTRI-GFP-pr1A #2 and subsequent penetration through the cuticle by (e) Ma2575 (f) *Af6982* (g) *Af6982con*^{ins} (h) *Af6982con*^{ins} pPTRI-GFP-pr1A #2. Cuticles were inoculated with 5×10^3 conidia and incubated 30°C for 24 h before staining with 0.01% Calcofluor white and viewing with fluorescence microscopy. Note the melanization of the cuticle at the location of the appressoria (a,b,c,d) and the area of hyphal penetration on the underside of the cuticle (e,f,g,h). *Af6982* pPTRI-GFP-pr1A #3 produced appressoria and penetrated the cuticle similar to the other *A. flavus* strains (data not shown).

Discussion

Employing *A. flavus* and *G. mellonella* as a pathogen-host model system for the study of pathogen evolution (Scully & Bidochka 2005; Scully & Bidochka 2006a), we isolated a strain, designated *Af6982con^{ins}*, displaying cysteine/methionine auxotrophy associated with difficulty growing on artificial media and a host range restriction due to an inability to complete its pathogenic cycle on plant but not insect hosts. This strain failed to grow on minimal media unless supplemented with cysteine or methionine and produced 100-fold fewer conidia than the wild type (*Af6982*) on PDA. Its host range was also diminished since it failed to conidiate on a variety of plants, but continued to infect and produce a sufficient quantity of conidia as the culmination of its pathogenic cycle on a number of insects. Thus, *Af6982con^{ins}* displayed characteristics of an obligate insect pathogen with no change in virulence compared to the wild-type opportunistic strain. We postulated that the nutrient requirement imposed a host restriction on *Af6982con^{ins}* that would provide the selection pressure for an increase in virulence. Furthermore, this may be a mechanism by which virulent obligate pathogens evolve (Scully & Bidochka 2006).

The purpose of this study was to experimentally test whether the expression of a virulence factor in a fungal strain that already displayed some characteristics of an obligate lifestyle could result in a virulent, obligate pathogen. To this end, we transformed *Af6982con^{ins}* with a subtilisin-like protease gene *pr1* from the facultative insect pathogen *M. anisopliae*. Although this virulence gene acquisition required genetic manipulation in a laboratory setting as opposed to evolution through natural processes, we felt that it was justifiable based on current theories of pathogen evolution. Virulent facultative and obligate pathogens evolve from opportunistic pathogens (Scheffer 1991) via horizontal virulence gene transfer, de novo mutation, and genome decay (Wren 2003) in conjunction with the natural selection provided during host infection (Brown et al. 2006). Among fungi, virulence factors may be acquired through de novo mutation, for example fungal toxins are postulated to have evolved by mutation

of secondary metabolite genes (Scheffer 1991), or by horizontal transfer, as was the case for *Pyrenophora tritici-repentis*, the causative agent of tan spot syndrome in wheat, which obtained its *ToxA* gene from *Stagonospora nodorum* (Friesen et al. 2006). Concerning *A. flavus*, the acquisition of an effective Pr1-like virulence factor could occur through de novo mutation of existing protease genes (St. Leger et al. 2000) or through horizontal gene transfer, a possibility posited by the close physical proximity of *M. anisopliae* and *A. flavus* as they both reside in the soil and infect insects (Hughes & Boomsma 2003). Regardless of the mechanism, pathogen evolution involves virulence gene acquisition, justifying the transformation of *Af6982con*^{ins} with Pr1 in this study.

Pr1 of *M. anisopliae* was chosen as the virulence factor for the transformation of *Af6982con*^{ins} because it is the most well-characterized virulence factor of entomopathogens. Consisting of 11 isoforms (A-H) (Bagga et al. 2004), Pr1 is a subtilisin-like, chymoelastinolytic, endoprotease (St. Leger et al. 1987a; St. Leger et al. 1992a), capable of degrading insect cuticle with tremendous efficiency (St. Leger et al. 1987a). Pr1 is the most abundant enzyme released from *M. anisopliae* appressoria (St. Leger et al. 1989b) and plays an essential role in degrading the proteinaceous component of cuticle, allowing the fungal pathogen to penetrate the insect (Clarkson & Charnley 1996). In addition, treatment with Pr1 inhibitors decreased *M. anisopliae* virulence towards *Manduca sexta* (St. Leger et al. 1988a), while *pr1A* and *pr1B*-deficient mutants exhibited a decreased lethality toward *Tenebrio molitor* (Wang et al. 2002).

Pr1 is unique among subtilisin-like proteases, including those produced by *A. flavus*, because it has a high pI (~10) (St. Leger et al. 1987a) due the large number of basic surface residues (St. Leger et al. 1992a). Consequently, Pr1 demonstrates an unusually high affinity for insect cuticle, adsorbing via non-specific electrostatic attraction to the abundant aspartic and glutamic acid residues. In fact, chemically altering the residues in the insect cuticle reduces the binding capacity of Pr1 (Bidochka & Khachatourians 1994). Presumably, the status of *M. anisopliae* as a facultative insect pathogen is due in part to the high affinity of Pr1 for insect

cuticle, which is an essential prerequisite for effective proteolytic degradation (Bidochka & Khachatourians 1994; St. Leger et al. 1986; St. Leger et al. 1991a). The cuticle binding assay highlights the difference between the *A. flavus* subtilisin-like proteases, of which only 25-30% adhere to insect cuticle following a water wash, and the entomopathogenic subtilisin-like protease Pr1, of which 66% adhere by electrostatic attraction that is disrupted only by washing with an ionic buffer (Figure 7-6). Since protease adsorption to insect cuticle is an essential step of proteolytic degradation and pathogenic penetration, we hypothesized that we could increase the virulence of *Af6982con*^{ins} towards insects by transforming it with Pr1A, the most abundant isoform (Bagga et al. 2004) of a subtilisin-like protease with a significantly higher affinity toward insect cuticle than native *A. flavus* subtilisins.

Af6982con^{ins} and *Af6982* were successfully transformed with Pr1. Integration of *pr1A* into the *A. flavus* genome was confirmed with Southern hybridization, while constitutive-Pr1 expression was substantiated with subtilisin-like enzyme assays and isoelectric focusing. In media containing abundant carbon, nitrogen (N-acetylglucosamine) and sulfur (methionine) sources known to suppress native subtilisin activity, all Pr1 transformants demonstrated constitutive subtilisin-like protease expression. Although both Pr1 transformants and non-transformants expressed subtilisin-like protease during growth in cuticle media, the Pr1-recombinants produced a mixture of both native proteases and Pr1 proteases as evidenced by cuticle binding assay values intermediate between those of *M. anisopliae* and *Af6982/Af6982con*^{ins} and IEF analysis which showed a high pI (~10) band in the samples of the *A. flavus* Pr1-recombinants identical to high pI (~10) band produced by Pr1 in the *M. anisopliae* sample (St. Leger et al. 1994b).

Despite the successful transformation of *A. flavus* with Pr1, virulence towards *G. mellonella* and *T. molitor* larvae was not affected. Virulence remained unchanged with both topical application, where Pr1 expression during growth on the insect cuticle could have potentially resulted in greater cuticle degradation and penetration, and injection, where

constitutive Pr1 expression in the highly nutritious insect hemolymph, which presumably suppresses native protease expression, could have degraded hemolymph proteins, disrupting the insect immune system response (Griesch & Vilcinskas 1998). There was no overall difference in virulence between the *Af6982con^{ins}* Pr-1 recombinants displaying characteristics of obligate insect pathogenesis and the Pr-1 transformants of the opportunist *Af6982*, while *M. anisopliae* remained significantly more virulent than any of the *A. flavus* strains. Furthermore, there was no change in appressoria formation, the penetration structures that produce Pr1 in *M. anisopliae* (Goettel et al. 1989), or cuticle penetration.

Although the recombinants constitutively expressed Pr1, they failed to overexpress Pr1. In cuticle media, *M. anisopliae* produced 10 times more subtilisin-like protease than any of the *A. flavus* strains. Hence, increased virulence may require enhanced levels of protease production of which *A. flavus* appears to be incapable. Since the *A. nidulans gpd* promoter functions well in *A. flavus* (Flaherty & Payne 1997) and is capable of causing Pr1 overexpression and increasing virulence of *M. anisopliae* toward *M. sexta* (St. Leger et al. 1996), the low levels of Pr1 in *A. flavus* may be due to limitations on protein processing or secretion. Low protein expression despite control by a constitutive promoter has been noted in other foreign gene expression systems (Wnendt et al. 1994; Zhang et al. 2006). Alternatively, perhaps an increase in insect virulence requires another virulence factor besides Pr1. The low virulence of *A. flavus* compared to *M. anisopliae* could be due to detrimental immune system recognition and clearance. Indeed, there is no report of *A. flavus* blastospores in the insect hemolymph (Kumar et al. 2004), a morphological structure known to avoid immune system recognition (Gillespie et al. 2000; Wang & St. Leger 2006). Toxin production may also be required since toxins such as destruxin are known to mediate *M. anisopliae* entomopathogenicity (Pal et al. 2007; Vey et al. 2002; Vilcinskas et al. 1997).

With respect to pathogen evolution and emerging diseases, the failure of Pr1 to increase the virulence of *A. flavus* emphasizes two points. First, the genetic background into which a

virulence factor is introduced must be conducive to its expression, processing, protection, and transport. The *A. flavus* genome appears to be incapable of expressing high levels of Pr1, implying that a favorable genetic background may be as important as virulence factor acquisition in pathogen evolution. Studies on the evolutionary history of known human pathogens, including MRSA *Staphylococcus aureus*, *Escherichia coli* OH157:H7, *Yersinia pestis*, and *Vibrio cholera*, emphasize the importance of genetic background in allowing virulence factor acquisition to instigate pathogen evolution (Brown et al. 2006; Mekalanos et al. 1997; Ochman & Moran 2001; Oliveria et al. 2002; Whittam et al. 1993; Wren 2003). Second, not all virulence factors are created equal. While the evolutionary paths of known human pathogens often point to dramatic increases in virulence following the acquisition of a single virulence factor (Friesen et al. 2006; Waldor & Mekalanos 1996; Waterfield et al. 2004; Whittam et al. 1993), our results confirm that transformation with a single virulence factor does not necessarily increase virulence. Other virulence factors may be required, or possibly an increase in virulence in some pathogens is evolutionarily impossible due to metabolic and genomic constraints.

GENERAL CONCLUSION

Although the etiological agents of many devastating diseases have been identified and effective treatment options employed, infectious pathogens are still a major cause of human death. In addition, a number of newly emerging diseases have recently been identified. Combating the emergence of novel diseases requires understanding the causative ecological and evolutionary factors. Studies interpreting inferences about the evolutionary histories of a number of current pathogens have identified three mechanisms of pathogen evolution: de novo mutation accompanying host association, genome decay, and virulence factor acquisition. In this thesis, I demonstrated that employing *A. flavus* infections in *G. mellonella* larvae as a model pathogen-host system allowed empirical observation and experimental manipulation of these three previously identified aspects of pathogen evolution. In addition to representing one of the few experimental systems designed to study pathogen evolution, it was unique in that it employed an opportunistic pathogen rather than a facultative pathogen, and thus investigates the evolution and emergence of novel diseases rather than simply an increase in virulence or host-specialization of a known pathogen.

The foundation of the study (Chapter 2) was the association of the opportunistic pathogen *A. flavus* with the insect host *G. mellonella* larvae whereby the fungus was serially propagated through insects 5 times. Following host association, both phenotypic and population genetic alterations were observed. These are considered de novo changes since the experimental design precluded the influx of other microorganisms or genetic elements. Phenotypically, *A. flavus* displayed some adaptations with respect to host colonization, but demonstrated a dramatic decrease in its ability to grow saprobically. Besides highlighting the types of phenotypic changes that can occur as a result of de novo mutations and host association, the results emphasized that the evolution of host specialization may rely much more heavily on the loss of an ability to grow on alternate hosts and substrates rather than an adaptation to the preferred host. Furthermore, although a pathogen population generates de novo mutations, Chapter 3 called

attention to the fact that passage through a host can dramatically alter the pathogen population structure. The use of amplified fragment length polymorphism as an estimate of the genetic diversity demonstrated that the host imposed a genetic bottleneck on the *A. flavus* population. Although the bottleneck was not an instance of positive selection, it nevertheless emphasized the host as a profound force in shaping pathogen population structure and directing its evolution.

The serial propagation of *A. flavus* through *G. mellonella* larvae also served to isolate a strain that displayed properties of an obligate insect pathogen, namely host restriction without a loss in virulence (Chapter 4). This strain was a cysteine/methionine auxotroph, indicating that its unique properties were the result of genome decay. Apparently, the insect host provided the required nutrients such that the loss the biosynthetic potential did not hinder pathogenesis. Although genome decay is a well-recognized component of pathogen evolution and many obligate pathogens rely on their hosts to obtain the necessary nutrients, this study recognized genome decay and the nutritional requirements of a pathogen as a potential mechanism of host restriction and selection pressure rather than an inconsequential by-product of host association. Although cysteine/methionine auxotrophy was attributed to an inability to reduce sulfate to sulfite, Chapter 5 noted that the genetic mutation did not reside in the APS kinase or PAPS reductase components of the sulfate assimilation pathway.

Beyond host restriction, Chapter 6 showed that cysteine/methionine auxotrophy also served to increase virulence factor production. Presumably in an attempt to gain usable cysteine or methionine from an exogenous protein source, the auxotroph secreted more protease, a virulence factor known to mediate insect pathogenesis (St. Leger 1995), than the wild-type when grown on media containing insect substrates intended to mimic the nutritional environment encountered during infection. Because the auxotroph also exhibited stunted growth on these media, the enhanced protease production apparently compensated for the stunted growth such that neither cuticle penetration nor virulence was diminished. Not only does this finding

highlight the evolution of potentially altered pathogenic strategy, it represents one of only a few cases where genome decay was shown to enhance a virulence component.

Horizontal transfer of virulence genes was pivotal in the emergence of a number of deadly diseases, including anthrax and plague. Therefore, interspecies gene transfer as a mechanism of pathogen evolution was investigated empirically using the fungal pathogen-insect host model system. In Chapter 7, the insect-restricted strain was transformed with Pr1, a protease known to mediate cuticle penetration and insect infection by the facultative insect pathogen *M. anisopliae*. Although *A. flavus* was successfully transformed and constitutively expressed Pr1, the amount of protease produced was exceedingly low compared to that excreted by *M. anisopliae*. This work emphasized the need for an apposite genetic background, specifically appropriate regulatory, post-transcriptional processing and modification, and secretion systems, in order for the horizontal transfer of virulence genes to affect pathogen evolution.

A. flavus infections in *G. mellonella* larvae provided an excellent pathogen-host model system, yielding insights into the mechanisms of pathogen evolution namely de novo mutation following host association, genome decay, and virulence factor acquisition. Hopefully, a greater understanding of pathogen evolution and its role in the emergence of novel diseases will be gained by further empirical investigation using this and other analogous pathogen-host model systems.

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APPENDICES

Appendix A

Appendix A demonstrated the experimental determination of the amount of UV light required to yield 80% kill of *A. flavus* conidia.

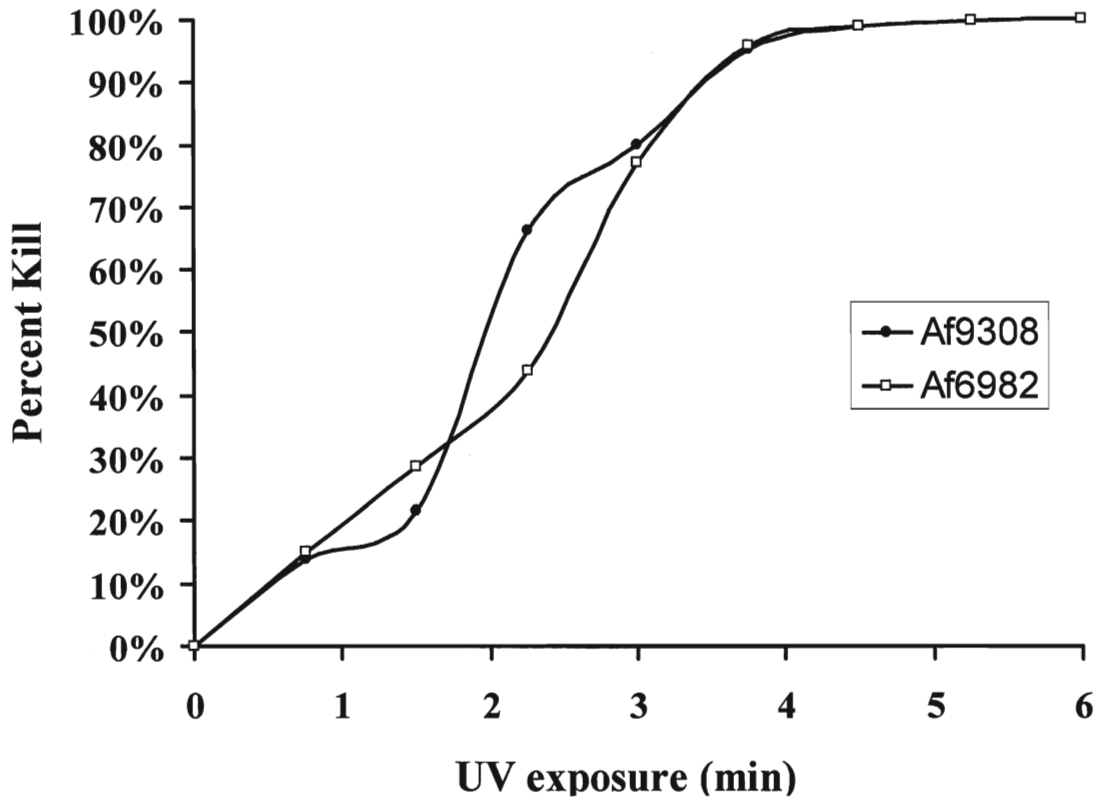


Figure A-1: Percent kill of *A. flavus* conidia following exposure to UV light ($400 \mu\text{W}/\text{cm}^2$) for varying lengths of time. Conidia suspension of 1×10^6 conidia/ml were exposed to UV light for 0, 0.75, 1.5, 2.25, 3.0, 3.75, 4.5, 5.25, and 6 min. For each time point of UV exposure, 100 μl of a 100-fold dilution of the mutagenized conidia was plated onto a Petri dish containing a 10ml of PDA. The number of colony forming units was counted, and the percent kill was calculated by comparing with the Petri dish not exposed to UV light.

Appendix B

Appendix B shows a summary of the linear regression analysis of the percent mortality, time to conidiation after death, number of conidia on the insect cadaver, and subculture-colony diameter during the serial propagation of *A. flavus* through *G. mellonella* larvae for 5 generations.

Table B-1: Summary of linear regression analyses showing the equation, the R-squared value, the F-value, and the P-value of the linear regression of the percent mortality of *G. mellonella* larvae during the serial propagation of *A. flavus* through the larvae for 5 generations.

Application	Strain	Lineage	Equation	R-squared	F-value	P-value
Topical	9308	1	$y = -0.041x + 0.414$	0.196	0.730	0.456
		2	$y = -0.229x + 0.750$	0.976	40.333	0.100
		3	$y = -0.015x + 0.361$	0.157	0.558	0.509
		4	$y = -0.114x + 0.669$	0.593	6.834	0.079
		5	$y = -0.063x + 0.448$	0.420	2.168	0.237
	6982	1	$y = -0.008x + 0.312$	0.005	0.014	0.914
		2	$y = -0.029x + 0.285$	0.197	0.737	0.454
		3	$y = -0.027x + 0.402$	0.031	0.096	0.777
		4	$y = -0.008x + 0.208$	0.011	0.033	0.868
		5	$y = 0.036x + 0.076$	0.277	1.151	0.362
	Double Infection	1	$y = 0.004x + 0.093$	0.0069	0.019	0.898
		2	$y = -0.012x + 0.220$	0.031	0.095	0.778
		3	$y = 0.019x + 0.121$	0.612	4.737	0.118
		4	$y = 0.088x - 0.035$	0.884	22.93	0.017*
		5	$y = 0.035x + 0.099$	0.267	1.095	0.372
	Mutated 9308	1	$y = 0.002x + 0.204$	0.002	0.007	0.939
		2	$y = -0.001x + 0.099$	0.001	0.001	0.980
		3	$y = 0.002x + 0.195$	0.017	0.051	0.836
		4	$y = 0.004x + 0.196$	0.009	0.028	0.878
		5	$y = -0.008x + 0.208$	0.030	0.094	0.779
	Mutated 6982	1	$y = -0.020x + 0.236$	0.521	1.119	0.368
		2	$y = 0.012x + 0.116$	0.188	0.692	0.466
		3	$y = 0.015x + 0.171$	0.137	0.475	0.540
		4	$y = 0.019x + 0.162$	0.217	0.833	0.429
		5	$y = 0.011x + 0.144$	0.058	0.184	0.697
Injection	9308	1	$y = -0.050x + 0.529$	0.230	0.896	0.414
		2	$y = 0.018x + 0.485$	0.104	0.033	0.867
	6982	1	$y = -0.027x + 0.582$	0.060	0.190	0.692
		2	$y = 0.095x + 0.226$	0.256	1.033	0.384
	Double Infection	1	$y = -0.089x + 0.667$	0.542	3.557	0.156
		2	$y = 0.005x + 0.363$	0.002	0.005	0.949
	Mutated 9308	1	$y = 0.033x + 0.307$	0.053	0.167	0.710
		2	$y = 0.076x + 0.159$	0.266	1.085	0.374
	Mutated 6982	1	$y = 0.062x + 0.116$	0.156	0.556	0.510
		2	$y = 0.111x + 0.130$	0.473	2.693	0.199

* indicates statistically significant P-values (<0.05).

Table B-2: Summary of linear regression analyses showing the equation, the R-squared value, the F-value, and the P-value of the linear regression of the number of days between the death of *G. mellonella* larvae and the conidiation of *A. flavus* on the cadavers over generations 1-5.

Application	Strain	Lineage	Equation	R-squared	F-value	P-value
Topical	9308	1	$y = 0.056x + 3.515$	0.002	0.012	0.916
		2	--	--	--	--
		3	$y = 0.440x + 1.436$	0.148	1.909	0.195
		4	$y = 0.214x + 2.058$	0.157	1.488	0.257
		5	$y = 1.313x + 0.500$	0.306	1.766	0.255
	6982	1	$y = -0.595x + 6.095$	0.263	2.493	0.158
		2	$y = 0.128x + 2.723$	0.032	0.165	0.702
		3	$y = -0.599x + 5.432$	0.421	6.541	0.031*
		4	$y = 0.311x + 2.107$	0.124	0.851	0.392
		5	$y = 0.011x + 3.326$	<0.001	0.001	0.977
	Double Infection	1	$y = -0.363x + 5.413$	0.128	0.735	0.430
		2	$y = -0.098x + 3.914$	0.019	0.170	0.690
		3	$y = -0.437x + 3.994$	0.169	2.234	0.163
		4	$y = -0.822x + 4.557$	0.460	9.366	0.011*
		5	$y = -0.532x + 4.706$	0.240	4.106	0.064
	Mutated 9308	1	$y = -0.232x + 3.377$	0.043	0.535	0.479
		2	$y = -0.288x + 4.088$	0.194	1.207	0.322
		3	$y = 0.217x + 1.874$	0.211	3.215	0.098
		4	$y = -0.180x + 4.044$	0.031	0.356	0.563
		5	$y = -0.315x + 4.335$	0.160	1.911	0.197
	Mutated 6982	1	$y = -0.448x + 4.859$	0.327	6.801	0.021*
		2	$y = -0.229x + 4.743$	0.027	0.282	0.607
		3	$y = -1.120x + 7.915$	0.527	10.017	0.011*
		4	$y = -0.660x + 5.425$	0.269	3.675	0.084
		5	$y = -0.278x + 4.823$	0.076	0.900	0.363
Injection	9308	1	$y = -0.184x + 2.478$	0.032	1.632	0.207
		2	$y = -0.033x + 2.178$	0.002	0.154	0.696
	6982	1	$y = -0.137x + 2.607$	0.025	1.590	0.212
		2	$y = 0.077x + 1.642$	0.015	0.896	0.347
	Double Infection	1	$y = -0.011x + 2.160$	0.001	0.007	0.933
		2	$y = 0.022x + 1.684$	0.002	0.099	0.754
	Mutated 9308	1	$y = -0.019x + 2.421$	0.001	0.006	0.935
		2	$y = 0.204x + 1.679$	0.014	0.588	0.447
	Mutated 6982	1	$y = -0.073x + 2.585$	0.004	0.139	0.712
		2	$y = 0.041x + 1.735$	0.002	0.087	0.769

* indicates statistically significant P-values (<0.05).

Table B-3: Summary of linear regression analyses showing the equation, the R-squared value, the F-value, and the P-value of the linear regression of the number of *A. flavus* conidia on the first insect to die and conidiate over generations 1-5 of *G. mellonella* larvae.

Application	Strain	Lineage	Equation	R-squared	F-value	P-value
Topical	9308	1	$y = -4.8 \times 10^7 x + 2.7 \times 10^8$	0.385	8.748	0.010*
		2	--	--	--	--
		3	$y = 6.6 \times 10^7 x + 2.0 \times 10^8$	0.219	3.361	0.092
		4	$y = 9.6 \times 10^7 x - 1.6 \times 10^8$	0.432	9.129	0.011*
		5	$y = -3.4 \times 10^7 x + 3.1 \times 10^8$	0.216	2.477	0.150
	6982	1	$y = -5.2 \times 10^6 x + 1.1 \times 10^8$	0.006	0.085	0.775
		2	$y = 1.0 \times 10^7 x - 2.3 \times 10^6$	0.184	2.708	0.126
		3	$y = 3.4 \times 10^7 x + 9.4 \times 10^6$	0.220	3.375	0.091
		4	$y = 1.1 \times 10^7 x - 1.0 \times 10^6$	0.606	21.490	<0.001*
		5	$y = 3.5 \times 10^7 x - 5.7 \times 10^7$	0.702	37.722	<0.001*
	Double Infection	1	$y = -2.3 \times 10^7 x + 1.7 \times 10^8$	0.345	9.500	0.006*
		2	$y = -3.0 \times 10^6 x + 1.6 \times 10^8$	0.005	0.078	0.783
		3	$y = 1.7 \times 10^7 x + 8.4 \times 10^7$	0.480	14.754	0.001*
		4	$y = 3.1 \times 10^7 x - 1.3 \times 10^7$	0.662	19.586	0.001*
		5	$y = 3.7 \times 10^7 x - 9.0 \times 10^6$	0.237	4.965	0.041*
	Mutated 9308	1	$y = 2.6 \times 10^7 x + 6.6 \times 10^7$	0.142	2.985	0.101
		2	$y = -6.1 \times 10^6 x + 8.7 \times 10^7$	0.042	0.696	0.416
		3	$y = 1.3 \times 10^7 x + 1.6 \times 10^7$	0.317	6.497	0.023*
		4	$y = -9.5 \times 10^6 x + 1.5 \times 10^8$	0.018	0.290	0.597
		5	$y = 1.4 \times 10^7 x + 3.4 \times 10^7$	0.298	5.085	0.044*
	Mutated 6982	1	$y = 5.4 \times 10^6 x + 3.1 \times 10^7$	0.038	0.639	0.436
		2	$y = -2.3 \times 10^5 x + 6.6 \times 10^6$	0.010	0.138	0.716
		3	$y = -1.3 \times 10^7 x + 1.4 \times 10^8$	0.199	3.987	0.063
		4	$y = 4.7 \times 10^7 x - 3.9 \times 10^7$	0.464	15.575	<0.001*
		5	$y = -1.8 \times 10^7 x + 1.2 \times 10^8$	0.221	5.105	0.036*
Injection	9308	1	$y = -1.5 \times 10^7 x + 3.0 \times 10^8$	0.066	1.268	0.275
		2	$y = 3.9 \times 10^6 x + 2.0 \times 10^8$	0.002	0.041	0.841
	6982	1	$y = 6.7 \times 10^6 x + 8.6 \times 10^7$	0.009	0.159	0.695
		2	$y = -7.8 \times 10^6 x + 2.1 \times 10^8$	0.047	0.890	0.358
	Double Infection	1	$y = 4.1 \times 10^5 x + 7.1 \times 10^7$	0.001	0.013	0.911
		2	$y = 1.3 \times 10^7 x + 8.0 \times 10^7$	0.051	0.976	0.336
	Mutated 9308	1	$y = 2.9 \times 10^7 x + 8.2 \times 10^7$	0.052	0.995	0.332
		2	$y = 5.5 \times 10^5 x + 1.3 \times 10^8$	0.001	0.005	0.946
	Mutated 6982	1	$y = -1.6 \times 10^7 x + 1.6 \times 10^8$	0.078	1.524	0.233
		2	$y = 2.0 \times 10^7 x + 8.1 \times 10^7$	0.109	2.211	0.154

* indicates statistically significant P-values (<0.05).

Table B-4: Summary of linear regression analyses showing the equation, the R-squared value, the F-value, and the P-value of the linear regression of the colony diameter of the *A. flavus* subcultures from the first insect to die and conidiate over generations 0-5 of *G. mellonella* larvae.

Application	Strain	Lineage	Equation	R-squared	F-value	P-value
Topical	9308	1	$y = -3.563x + 72.082$	0.546	13.246	0.004*
		2	--	--	--	--
		3	$y = -2.686x + 70.912$	0.647	21.989	0.001*
		4	$y = -0.205x + 68.260$	0.785	47.349	<0.001*
		5	$y = -1.832x + 69.556$	0.540	11.757	0.007*
	6982	1	$y = -1.337x + 65.898$	0.722	31.090	<0.001*
		2	$y = -2.466x + 66.841$	0.802	52.746	<0.001*
		3	$y = -1.501x + 67.203$	0.505	12.221	0.004*
		4	$y = -1.600x + 66.656$	0.789	44.968	<0.001*
		5	$y = -1.401x + 66.435$	0.473	10.782	0.007*
	Double Infection	1	$y = -2.784x + 69.686$	0.823	55.646	<0.001*
		2	$y = -2.433x + 69.337$	0.619	21.149	<0.001*
		3	$y = -1.200x + 67.373$	0.138	2.086	0.172
		4	$y = -1.461x + 65.972$	0.464	11.234	0.005*
		5	$y = -1.333x + 65.717$	0.307	5.764	0.032*
	Mutated 9308	1	$y = -1.577x + 67.232$	0.561	19.180	<0.001*
		2	$y = -1.660x + 69.404$	0.720	41.185	<0.001*
		3	$y = -2.157x + 67.933$	0.735	44.324	<0.001*
		4	$y = -2.367x + 70.071$	0.584	22.471	<0.001*
		5	$y = -2.446x + 69.052$	0.939	231.356	<0.001*
	Mutated 6982	1	$y = -2.148x + 66.786$	0.735	38.756	<0.001*
		2	$y = -1.790x + 63.744$	0.586	16.979	0.001*
		3	$y = -2.199x + 63.924$	0.699	34.875	<0.001*
		4	$y = -1.368x + 66.651$	0.633	27.570	<0.001*
		5	$y = -1.460x + 67.474$	0.159	2.830	0.113
Injection	9308	1	$y = -2.301x + 70.321$	0.555	18.714	<0.001*
		2	$y = -1.099x + 67.551$	0.356	7.194	0.019*
	6982	1	$y = -1.206x + 67.396$	0.631	22.225	<0.001*
		2	$y = -0.775x + 65.477$	0.263	5.339	0.035*
	Double Infection	1	$y = -1.148x + 66.588$	0.339	6.657	0.023*
		2	$y = -0.980x + 66.614$	0.216	3.304	0.094
	Mutated 9308	1	$y = -0.612x + 64.953$	0.049	0.667	0.429
		2	$y = -1.420x + 66.202$	0.317	7.427	0.015*
	Mutated 6982	1	$y = -1.456x + 62.946$	0.109	1.949	0.182
		2	$y = -0.614x + 64.258$	0.129	2.370	0.143

* indicates statistically significant P-values (<0.05).

Appendix C

Appendix C shows a summary of the linear regression analysis of the subculture-colony diameter during the serial subculturing of *A. flavus* on PDA for 15 subcultures.

Table C-1: Summary of linear regression analyses showing the equation, the R-squared value, the F-value, and the P-value of the linear regression of the colony diameter of *A. flavus* serially subcultured on PDA for 15 subcultures.

Strain	Lineage	Equation	R-squared	F-value	P-value
9308	1	$y = 0.220x + 63.374$	0.072	0.934	0.353
	2	$y = -0.459x + 68.103$	0.279	5.041	0.043*
	3	$y = 0.369x + 63.372$	0.206	3.368	0.089
	4	$y = -0.148x + 66.591$	0.122	1.528	0.242
	5	$y = -0.292x + 68.455$	0.046	0.622	0.445
6982	1	$y = 0.096x + 63.657$	0.049	0.563	0.469
	2	$y = 0.221x + 62.952$	0.084	1.012	0.336
	3	$y = 0.213x + 61.851$	0.257	4.144	0.064
	4	$y = 0.081x + 64.265$	0.070	0.902	0.361
	5	$y = 0.022x + 63.268$	0.001	0.004	0.953

* indicates statistically significant P-values (<0.05).

Appendix D shows the amount of conidia produced by *Af6982con^{ins}* relative to *Af6982* on YPD, NA, PDA, and *G. mellonella* larvae for generations 2-6 during serial passage through *G. mellonella* larvae.

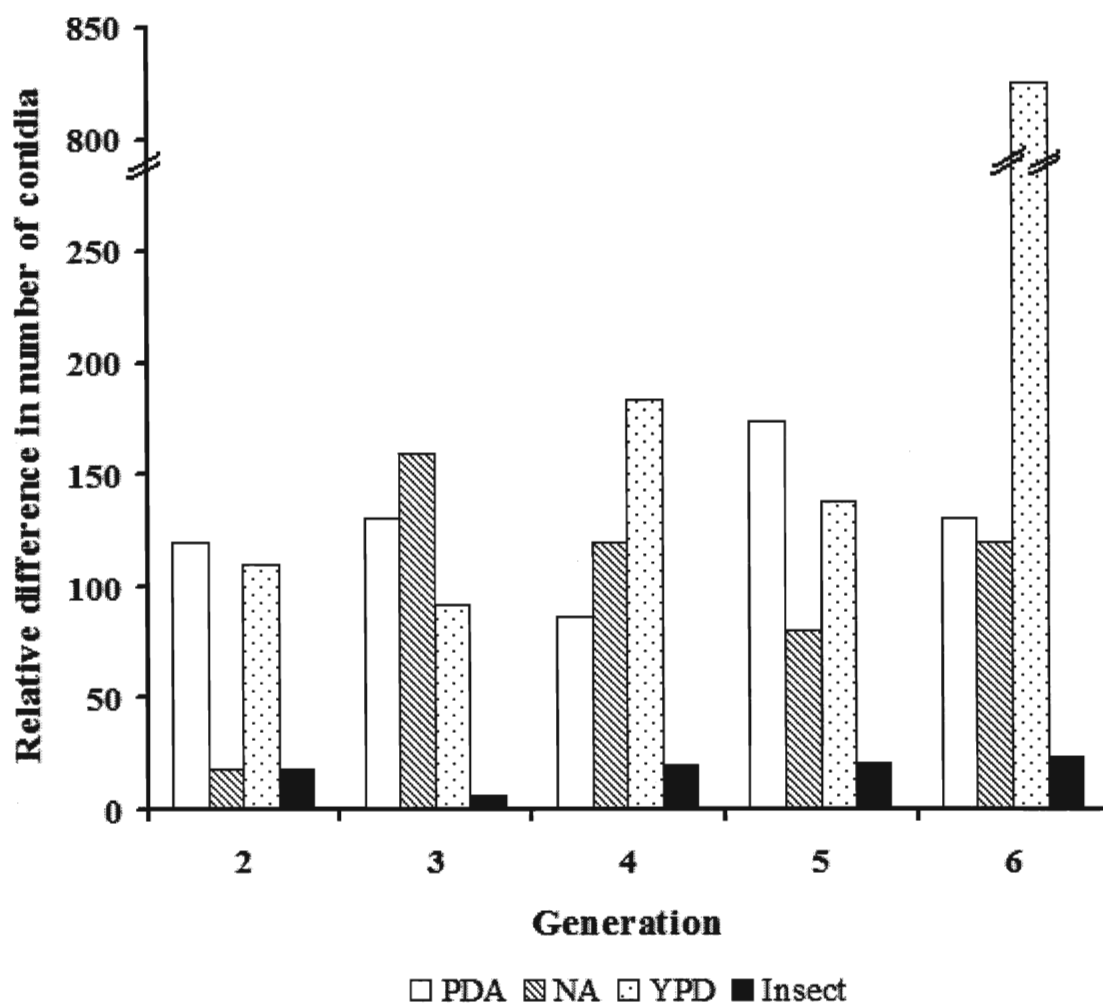


Figure D-1: Relative difference in number of conidia produced by *Af6982* and *Af6982con^{ins}* on potato dextrose agar (PDA), nutrient agar (NA), yeast peptone dextrose agar (YPD), and *G. mellonella* larvae (insect) during generations 2-6 of serial passage through *G. mellonella* larvae. The relative difference was calculated as the number of conidia produced by *Af6982* divided by the number of conidia produced by *Af6982con^{ins}*. The amount of conidia on *G. mellonella* larvae was assessed after topical application ($5\mu\text{l}$ of 1×10^5 conidia ml^{-1}) during the serial passage. For PDA, NA, and YPD, the amount of conidia was calculated as the mean of three replicates. The conidial counts for *G. mellonella* larvae were taken from the insect from which the conidia were harvested for infection of the next generation of *G. mellonella* larvae.

Appendix E

The internal transcribed spacer (ITS) sequence of *Af6982con*^{ins} (Figure E-1) demonstrated 97% similarity with the ITS sequences of *A. flavus* and *A. oryzae*. *A. flavus* and *A. oryzae* are highly related such that the ITS sequence is unable to distinguish these two species. In fact, some taxonomists argue that they should be considered the same species. The primary distinguishing feature between the two species is that many *A. flavus* strains produce aflatoxins, while *A. oryzae* strains never produce aflatoxins. Given the highly toxic nature of aflatoxins and the industrial use of *A. oryzae* in soy sauce fermentation, *A. flavus* and *A. oryzae* are considered separate species (Campbell 2004). Thus, although the ITS of *Af6982con*^{ins} is highly similar to *A. oryzae*, it is considered an *A. flavus* strain derived from *Af6982*.

	10	20	30	40
<i>A. flavus 6982conins</i> (AY521473)	AA~GATGATTACCGAGCTGAAGGGTTTCCTAGCGAGCCCAA			
<i>A. oryzae</i> (AY373857)	AAGGATCATTACCGAG~TGTAGGGTTTCCTAGCGAGCCCAA			
<i>A. flavus</i> (AY373848)	AAGGATCATTACCGAG~TGTAGGGTTTCCTAGCGAGCCCAA			
<i>A. oryzae</i> (AF459735)	AAGGATCATTACCGAG~TGTAGGGTTTCCTAGCGAGCCCAA			
<i>A. flavus</i> (AF027863)	AAGGATCATTACCGAG~TGTAGGGTTTCCTAGCGAGCCCAA			
<i>A. flavus</i> (AF138287)	AAGGATCATTACCGAG~TGTAGGGTTTCCTAGCGAGCCCAA			
	50	60	70	80
<i>A. flavus 6982conins</i> (AY521473)	CCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG			
<i>A. oryzae</i> (AY373857)	CCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG			
<i>A. flavus</i> (AY373848)	CCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG			
<i>A. oryzae</i> (AF459735)	CCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG			
<i>A. flavus</i> (AF027863)	CCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG			
<i>A. flavus</i> (AF138287)	CCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGG			
	90	100	110	120
<i>A. flavus 6982conins</i> (AY521473)	GCCCGCCATTTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC			
<i>A. oryzae</i> (AY373857)	GCCCGCCATTTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC			
<i>A. flavus</i> (AY373848)	GCCCGCCATTTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC			
<i>A. oryzae</i> (AF459735)	GCCCGCCATTTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC			
<i>A. flavus</i> (AF027863)	GCCCGCCATTTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC			
<i>A. flavus</i> (AF138287)	GCCCGCCATTTCATGGCCGCCGGGGGCTCTCAGCCCCGGGC			
	130	140	150	160
<i>A. flavus 6982conins</i> (AY521473)	CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG			
<i>A. oryzae</i> (AY373857)	CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG			
<i>A. flavus</i> (AY373848)	CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG			
<i>A. oryzae</i> (AF459735)	CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG			
<i>A. flavus</i> (AF027863)	CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG			
<i>A. flavus</i> (AF138287)	CCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAG			
	170	180	190	200
<i>A. flavus 6982conins</i> (AY521473)	TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACCTTT			
<i>A. oryzae</i> (AY373857)	TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACCTTT			
<i>A. flavus</i> (AY373848)	TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACCTTT			
<i>A. oryzae</i> (AF459735)	TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACCTTT			
<i>A. flavus</i> (AF027863)	TGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACCTTT			
<i>A. flavus</i> (AF138287)	TGAANTCTGAGTTGATTGTATCGCAATCAGTTAAAACCTTT			

	210	220	230	240
			
<i>A. flavus</i> 6982conins (AY521473)	CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC			
<i>A. oryzae</i> (AY373857)	CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC			
<i>A. flavus</i> (AY373848)	CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC			
<i>A. oryzae</i> (AF459735)	CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC			
<i>A. flavus</i> (AF027863)	CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC			
<i>A. flavus</i> (AF138287)	CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC			

	250	260	270	280
			
<i>A. flavus</i> 6982conins (AY521473)	AGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCGGTG			
<i>A. oryzae</i> (AY373857)	AGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCGGTG			
<i>A. flavus</i> (AY373848)	AGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCGGTG			
<i>A. oryzae</i> (AF459735)	AGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCGGTG			
<i>A. flavus</i> (AF027863)	AGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCGGTG			
<i>A. flavus</i> (AF138287)	AGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCGGTG			

	290	300	310	320
			
<i>A. flavus</i> 6982conins (AY521473)	AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT			
<i>A. oryzae</i> (AY373857)	AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT			
<i>A. flavus</i> (AY373848)	AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT			
<i>A. oryzae</i> (AF459735)	AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT			
<i>A. flavus</i> (AF027863)	AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT			
<i>A. flavus</i> (AF138287)	AATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT			

	330	340	350	360
			
<i>A. flavus</i> 6982conins (AY521473)	TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATC			
<i>A. oryzae</i> (AY373857)	TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATC			
<i>A. flavus</i> (AY373848)	TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATC			
<i>A. oryzae</i> (AF459735)	TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATC			
<i>A. flavus</i> (AF027863)	TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATC			
<i>A. flavus</i> (AF138287)	TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATC			

	370	380	390	400
			
<i>A. flavus</i> 6982conins (AY521473)	AAGCACGGCTTGTGTGTTGGGTCGTCGTCGCCCTCTCCGGC			
<i>A. oryzae</i> (AY373857)	AAGCACGGCTTGTGTGTTGGGTCGTCGTCGCCCTCTCCGGG			
<i>A. flavus</i> (AY373848)	AAGCACGGCTTGTGTGTTGGGTCGTCGTCGCCCTCTCCGGG			
<i>A. oryzae</i> (AF459735)	AAGCACGGCTTGTGTGTTGGGTCGTCGTCGCCCTCTCCGGG			
<i>A. flavus</i> (AF027863)	AAGCACGGCTTGTGTGTTGGGTCGTCGTCGCCCTCTCCGGG			
<i>A. flavus</i> (AF138287)	AAGCACGGCTTGTGTGTTGGGTCGTCGTCGCCCTCTCCGGG			

	410	420	430	440
			
<i>A. flavus</i> 6982conins (AY521473)	GGGGACGGGTCCCAAAGGCAGCAGCGGCACCGCGTCCGAT			
<i>A. oryzae</i> (AY373857)	GGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT			
<i>A. flavus</i> (AY373848)	GGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT			
<i>A. oryzae</i> (AF459735)	GGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT			
<i>A. flavus</i> (AF027863)	GGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT			
<i>A. flavus</i> (AF138287)	GGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGAT			
	450	460	470	480
			
<i>A. flavus</i> 6982conins (AY521473)	CCTCGAGCGAATGGGGCTTTGACACCCGCTCTGAAGGCCC			
<i>A. oryzae</i> (AY373857)	CCTCGAGCGTATGGGGCTTTGTACCCGCTCTGTAGGCCC			
<i>A. flavus</i> (AY373848)	CCTCGAGCGTATGGGGCTTTGTACCCGCTCTGTAGGCCC			
<i>A. oryzae</i> (AF459735)	CCTCGAGCGTATGGGGCTTTGTACCCGCTCTGTAGGCCC			
<i>A. flavus</i> (AF027863)	CCTCGAGCGTATGGGGCTTTGTACCCGCTCTGTAGGCCC			
<i>A. flavus</i> (AF138287)	CCTCGAGCGTATGGGGCTTTGTACCCGCTCTGTAGGCCC			
	490	500	510	520
			
<i>A. flavus</i> 6982conins (AY521473)	GGACGGTGCTTGACGAACGCAAATCAATCTTTTTCCAGGT			
<i>A. oryzae</i> (AY373857)	GGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGT			
<i>A. flavus</i> (AY373848)	GGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGT			
<i>A. oryzae</i> (AF459735)	GGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGT			
<i>A. flavus</i> (AF027863)	GGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGT			
<i>A. flavus</i> (AF138287)	GGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGT			
	530	540	550	560
			
<i>A. flavus</i> 6982conins (AY521473)	TGACCTCGGATCATGTACGGATACCCGCTGAACCTAAGCA			
<i>A. oryzae</i> (AY373857)	TGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCA			
<i>A. flavus</i> (AY373848)	TGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCA			
<i>A. oryzae</i> (AF459735)	TGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCA			
<i>A. flavus</i> (AF027863)	TGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCA			
<i>A. flavus</i> (AF138287)	TGACCTCGGATCAGGTAGGGATACCCGCTGAACCTAAGCA			
	570			
			
<i>A. flavus</i> 6982conins (AY521473)	TATCAATC~GCGA			
<i>A. oryzae</i> (AY373857)	TATCAATAAGCGG			
<i>A. flavus</i> (AY373848)	TATCAATAAGCGG			
<i>A. oryzae</i> (AF459735)	TATCAATAAGCGG			
<i>A. flavus</i> (AF027863)	TATCAATAAGCGG			
<i>A. flavus</i> (AF138287)	TATCAATAAGCGG			

Figure E-1: The nucleotide sequence alignment of the internal transcribed spacer (ITS) region from *A. flavus* 6982con^{ins} aligned with the top five most similar GenBank sequences as determined by BLASTN. The GenBank accession number is listed in parentheses following the species name. “.” indicates the same nucleotide as *A. flavus* 6982con^{ins}; “~” indicates a gap.

Appendix F

Appendix F shows the auxanographic tests that confirmed the phenotypes of various *A. flavus* auxotrophs described by Papa (1980).

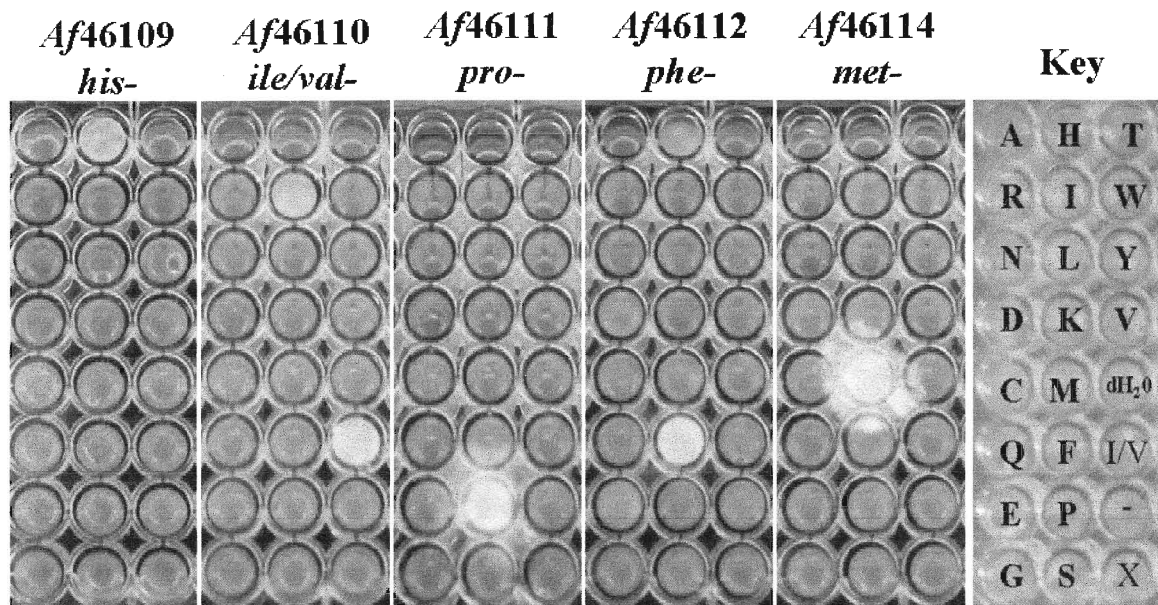


Figure F-1: Growth of *A. flavus* strains *Af46109*, *Af46110*, *Af46111*, *Af46112*, and *Af46114* on Cz media supplemented with various amino acids. Wells containing 200 μ l of Cz agar were supplemented with 10mM of the amino acid indicated in the key. "I/V" indicates equal amounts of isoleucine and valine (5mM each). All wells except "X" were inoculated with 5 μ l of a 1×10^6 conidia ml⁻¹ and incubated at 30°C for 3 d.

Appendix G

Appendix G displays the APS kinase and PAPS reductase open reading frame sequences of *Af6982* and *Af6982con^{ins}* aligned with sequences from other *Aspergillus* spp.

	10	20	30	40
<i>A. flavus6982 gene (EU016184)</i>	ATGTCCACGTAAGTCTATACATCCTTCCCCTCCCCACCA		
<i>Af6982conins gene (EU016185)</i>	ATGTCCACGTAAGTCTATACATCCTTCCCCTCCCCACCA		
<i>A. nidulans sD gene (Y08866)</i>	ATGGCCACGTGGG~~~~~ATCCCCATCCTCTTACACAA		
<i>A. nidulans mRNA (XM_653706)</i>	ATGGCCAC~~~~~		
<i>A. fumigatus mRNA (XM_747354.1)</i>	ATGGCCAC~~~~~		
<i>A. clavatus mRNA (XM_001269178)</i>	ATGGCCAC~~~~~		
	50	60	70	80
<i>A. flavus6982 gene (EU016184)</i>	TGCAGATACCCCTCCATCCCGCAGCCACTAACATAATCAC		
<i>Af6982conins gene (EU016185)</i>	TGCAGATACCCCTCCATCCCGCAGCCACTAACATAATCAC		
<i>A. nidulans sD gene (Y08866)</i>	T~~~~~CCACCGTTAACTAATCAAGCCCCCAT		
<i>A. nidulans mRNA (XM_653706)</i>	~~~~~		
<i>A. fumigatus mRNA (XM_747354.1)</i>	~~~~~		
<i>A. clavatus mRNA (XM_001269178)</i>	~~~~~		
	90	100	110	120
<i>A. flavus6982 gene (EU016184)</i>	CAAAAGAAACATCACCTTCCATGCCAGCGCCCTGACGCGC		
<i>Af6982conins gene (EU016185)</i>	CAAAAGAAACATCACCTTCCATGCCAGCGCCCTGACGCGC		
<i>A. nidulans sD gene (Y08866)</i>	TTATAGAAACATCACCCACCACGCCGGC~~~CTCACGCGC		
<i>A. nidulans mRNA (XM_653706)</i>	~~~~~AAACATCACCCACCACGCCGGC~~~ATCACGCGC		
<i>A. fumigatus mRNA (XM_747354.1)</i>	~~~~~AAACATCACCTACCACGCCAGCGCCCTCACCCGC		
<i>A. clavatus mRNA (XM_001269178)</i>	~~~~~CAACATCACCTACCACGCCAGCGCCCTCACGCGC		
	130	140	150	160
<i>A. flavus6982 gene (EU016184)</i>	AGCGAACGCAGCGAACTCCGCAACCAACGCGGTCTCACAA		
<i>Af6982conins gene (EU016185)</i>	AGCGAACGCAGCGAACTCCGCAACCAACGCGGTCTCACAA		
<i>A. nidulans sD gene (Y08866)</i>	AATGAGCGCAACCAGCTCCGCAAGCAGAAAGGCCTCACAA		
<i>A. nidulans mRNA (XM_653706)</i>	AATGAGCGCAACCAGCTCCGCAAGCAGAAAGGCCTCACAA		
<i>A. fumigatus mRNA (XM_747354.1)</i>	TCCGAGCGCAGCGCCCTCCGAAACCAACGCGGCCTCACAA		
<i>A. clavatus mRNA (XM_001269178)</i>	TCCGAGCGCAGCGCCCTCCGCAACCAGCGCGGCCTCACAA		
	170	180	190	200
<i>A. flavus6982 gene (EU016184)</i>	TCTGGCTCACCGGTCTCTCCGCCTCGGGCAAGTCTACCAT		
<i>Af6982conins gene (EU016185)</i>	TCTGGCTCACCGGTCTCTCCGCCTCGGGCAAGTCTACCAT		
<i>A. nidulans sD gene (Y08866)</i>	TATGGTTGACCGGCCTCTCCGCCTCCGGCAAGTCTACCAT		
<i>A. nidulans mRNA (XM_653706)</i>	TATGGTTGACCGGCCTCTCCGCCTCCGGCAAGTCTACCAT		
<i>A. fumigatus mRNA (XM_747354.1)</i>	TCTGGCTCACCGGCCTCTCCGCCTCGGGTAAATCCACCAT		
<i>A. clavatus mRNA (XM_001269178)</i>	TCTGGCTCACCGGCCTCTCAGCCTCCGGCAAATCGACCAT		
	210	220	230	240
<i>A. flavus6982 gene (EU016184)</i>	TGCCGTTGAGCTCGAGCACCAGCTCCTCCGAGACCGGGGT		
<i>Af6982conins gene (EU016185)</i>	TGCCGTTGAGCTCGAGCACCAGCTCCTCCGAGACCGGGGT		
<i>A. nidulans sD gene (Y08866)</i>	TGCCGTTGAGCTGGAGCACCAGCTTCTCC~~~AGCGCGGT		
<i>A. nidulans mRNA (XM_653706)</i>	TGCCGTTGAGCTGGAGCACCAGCTTCTCC~~~AGCGCGGT		
<i>A. fumigatus mRNA (XM_747354.1)</i>	CGCCGTCGAACTCGAGCACCAGCTGCTCCGCGACCGGGGC		
<i>A. clavatus mRNA (XM_001269178)</i>	CGCCGTCGAGCTCGAGCACCAGCTGCTGCGCGACCGCGGC		

	250	260	270	280
			
<i>A. flavus</i> 6982 gene(EU016184)	GTCCACGCCTACCGTCTCGACGGTGACAACATCCGCTTCG			
<i>Af6982conins</i> gene(EU016185)	GTCCACGCCTACCGTCTCGACGGTGACAACATCCGCTTCG			
<i>A. nidulans</i> sD gene(YO8866)	CTGCACGCCTACCGCCTCGACGGCGACAACGTGCGCTTCG			
<i>A. nidulans</i> mRNA(XM_653706)	CTGCACGCCTACCGCCTCGACGGCGACAACGTGCGCTTCG			
<i>A. fumigatus</i> mRNA(XM_747354.1)	GTCTCCGCCTACCGACTCGACGGCGACAACATCCGCTTCG			
<i>A. clavatus</i> mRNA(XM_001269178)	GTCGCCGCCTACCGCCTCGACGGCGATAACATCCGCTTCG			
	290	300	310	320
			
<i>A. flavus</i> 6982 gene(EU016184)	GACTCAACAAGGACCTCGGTTTTAGCGAAAAGGACCGCAA			
<i>Af6982conins</i> gene(EU016185)	GACTCAACAAGGACCTCGGTTTTAGCGAAAAGGACCGCAA			
<i>A. nidulans</i> sD gene(YO8866)	GCCTGAACAAAGATCTCGGTTTCTCCGACGCTGATCGCAA			
<i>A. nidulans</i> mRNA(XM_653706)	GCCTGAACAAAGATCTCGGTTTCTCCGACGCTGATCGCAA			
<i>A. fumigatus</i> mRNA(XM_747354.1)	GGCTCAACAAGGACCTAGGTTTCAGCGAAAAGGACCGCAA			
<i>A. clavatus</i> mRNA(XM_001269178)	GCCTGAACAAGGACCTGGGCTTCAGCGAAGCCGACCGCAA			
	330	340	350	360
			
<i>A. flavus</i> 6982 gene(EU016184)	CGAGAACATCCGTCGGATTGCAGAGGTTGCCAAGCTCTTC			
<i>Af6982conins</i> gene(EU016185)	CGAGAACATCCGTCGGATTGCAGAGGTTGCCAAGCTCTTC			
<i>A. nidulans</i> sD gene(YO8866)	TGAGAACATCCGGCGCATTGCAGAAGTCGCGAAGCTCTTC			
<i>A. nidulans</i> mRNA(XM_653706)	TGAGAACATCCGGCGCATTGCAGAAGTCGCGAAGCTCTTC			
<i>A. fumigatus</i> mRNA(XM_747354.1)	CGAGAACATCCGGCGCATTGCAGAAGTAGCCAAGCTCTTC			
<i>A. clavatus</i> mRNA(XM_001269178)	CGAGAACATCCGCCGCATCGCCGAGGTCGCCAAGCTGTTT			
	370	380	390	400
			
<i>A. flavus</i> 6982 gene(EU016184)	GCCGACAGCGCCTCTATCGCCATCACCTCGTTCATCTCGC			
<i>Af6982conins</i> gene(EU016185)	GCCGACAGCGCCTCTATCGCCATCACCTCGTTCATCTCGC			
<i>A. nidulans</i> sD gene(YO8866)	GCTGACAGTTCTCTCAATCGCCATCACGAGTTTCATCTCTC			
<i>A. nidulans</i> mRNA(XM_653706)	GCTGACAGTTCTCTCAATCGCCATCACGAGTTTCATCTCTC			
<i>A. fumigatus</i> mRNA(XM_747354.1)	GCCGACAGCGCCAACATCGCCATCACGTCGTTTATCTCGC			
<i>A. clavatus</i> mRNA(XM_001269178)	GCCGACAGCGCCAGCATCGCCATCACCTCCTTCATCTCGC			
	410	420	430	440
			
<i>A. flavus</i> 6982 gene(EU016184)	CCTACCGTGACAGACCGTGACACCGCGCGAAACTGCACGA			
<i>Af6982conins</i> gene(EU016185)	CCTACCGTGACAGACCGTGACACCGCGCGAAACTGCACGA			
<i>A. nidulans</i> sD gene(YO8866)	CTTTCCGGGCTGACCGTGATACCGCGCGAAACTGCACGA			
<i>A. nidulans</i> mRNA(XM_653706)	CTTTCCGGGCTGACCGTGATACCGCGCGAAACTGCACGA			
<i>A. fumigatus</i> mRNA(XM_747354.1)	CGTACAAGGCTGACCGCGAGACTGCGCGCAAGCTGCACGA			
<i>A. clavatus</i> mRNA(XM_001269178)	CTTACAAGGCGGACCGCGACACCGCCCGCAGGCTGCACGA			
	450	460	470	480
			
<i>A. flavus</i> 6982 gene(EU016184)	AGTCCCCACCCCGGGTGAAGAGACCGGTTTGCCCTTCGTT			
<i>Af6982conins</i> gene(EU016185)	AGTCCCCACCCCGGGTGAAGAGACCGGTTTGCCCTTCGTT			
<i>A. nidulans</i> sD gene(YO8866)	GGTCCCTACGCCGAATGATAGCACTGGTTTGCCATTTGTC			
<i>A. nidulans</i> mRNA(XM_653706)	GGTCCCTACGCCGAATGATAGCACTGGTTTGCCATTTGTC			
<i>A. fumigatus</i> mRNA(XM_747354.1)	GGTGCCGACGCCTGGCGAGGAGACGGGTCTGCCTTTTCGTC			
<i>A. clavatus</i> mRNA(XM_001269178)	GGTCCCCACCCCGGGCGAGGAGTCCGGTCTCCCTTCGTC			

	490	500	510	520
			
<i>A. flavus</i> 6982 gene(EU016184)	GAGGTCTTCATCGATGTCCCCATTGAGGTTGCCGAGCAGC			
<i>Af6982conins</i> gene(EU016185)	GAGGTCTTCATCGATGTCCCCATTGAGGTTGCCGAGCAGC			
<i>A. nidulans</i> sD gene(YO8866)	GAGGTATTCTGTTGATGTGCCGATTGAGGTTGCTGAGAAGA			
<i>A. nidulans</i> mRNA(XM_653706)	GAGGTATTCTGTTGATGTGCCGATTGAGGTTGCTGAGAAGA			
<i>A. fumigatus</i> mRNA(XM_747354.1)	GAGGTGTATGTCTGATGTGCCAGTGGAGGTGGCAGAGAAGC			
<i>A. clavatus</i> mRNA(XM_001269178)	GAGGTCTATGTCTGATGTCCCCGTTGAGGTGGCTGAGCAGC			
	530	540	550	560
			
<i>A. flavus</i> 6982 gene(EU016184)	GTGACCCCAAGGGTCTTTATAAGTTGGCCAGGGCGGGTAA			
<i>Af6982conins</i> gene(EU016185)	GTGACCCCAAGGGTCTTTATAAGTTGGCCAGGGCGGGTAA			
<i>A. nidulans</i> sD gene(YO8866)	GAGATCCCAAGGGGTTGTACAAGAAGGCCCGTGAGGGAAT			
<i>A. nidulans</i> mRNA(XM_653706)	GAGATCCCAAGGGGTTGTACAAGAAGGCCCGTGAGGGAAT			
<i>A. fumigatus</i> mRNA(XM_747354.1)	GGGATCCCAAGGGGCTGTATAAGAAGGCAAGGGAGGGGGT			
<i>A. clavatus</i> mRNA(XM_001269178)	GCGACCCCAAGGGTCTGTATAAGAAGGCGCGTGAGGGTAT			
	570	580	590	600
			
<i>A. flavus</i> 6982 gene(EU016184)	GATTTTCGGAGTTCACCGGCATCAGTGCGCCTTACGAGGAA			
<i>Af6982conins</i> gene(EU016185)	GATTTTCGGAGTTCACCGGCATCAGTGCGCCTTACGAGGAA			
<i>A. nidulans</i> sD gene(YO8866)	CATCAAGGAGTTCACGGGTATTAGCAGTCCGTATGAGGCG			
<i>A. nidulans</i> mRNA(XM_653706)	CATCAAGGAGTTCACGGGTATTAGCAGTCCGTATGAGGCG			
<i>A. fumigatus</i> mRNA(XM_747354.1)	CATCAAGGAGTTTACCGGGATTAGTGCTCCGTATGAGGCA			
<i>A. clavatus</i> mRNA(XM_001269178)	CATCAAGGAGTTCACCGGGATTAGTGCCCCGTATGAGGCG			
	610	620	630	640
			
<i>A. flavus</i> 6982 gene(EU016184)	CCTGAAAAGCCTGAGGTGCATATCCATAACCATGATTTGC			
<i>Af6982conins</i> gene(EU016185)	CCTGAAAAGCCTGAGGTGCATATCCATAACCATGATTTGC			
<i>A. nidulans</i> sD gene(YO8866)	CCTGAAAACCCGGAGGTTTCATGTGAAGAATGTGGATCTAC			
<i>A. nidulans</i> mRNA(XM_653706)	CCTGAAAACCCGGAGGTTTCATGTGAAGAATGTGGATCTAC			
<i>A. fumigatus</i> mRNA(XM_747354.1)	CCGGATAAGCCCGAGGTGCATATCAAGAACTATGATTTGC			
<i>A. clavatus</i> mRNA(XM_001269178)	CCGGATAAGCCCGAGGTGCATATCAAGAACTATGACTTGC			
	650	660	670	680
			
<i>A. flavus</i> 6982 gene(EU016184)	CAGTCCAGGATGCTGTGAAGCAGATTGTGGATTATTTGGA			
<i>Af6982conins</i> gene(EU016185)	CAGTCCAGGATGCTGTGAAGCAGATTGTGGATTATTTGGA			
<i>A. nidulans</i> sD gene(YO8866)	CGATTTCAGGAGGCTGTCAAGCAGATTATTGACTACTTGGA			
<i>A. nidulans</i> mRNA(XM_653706)	CGATTTCAGGAGGCTGTCAAGCAGATTATTGACTACTTGGA			
<i>A. fumigatus</i> mRNA(XM_747354.1)	CCGTGAGGGATGCGGTTGCGCAGATCATTGCGTATCTGGA			
<i>A. clavatus</i> mRNA(XM_001269178)	CTGTGAAGGATGCCGTGGCGCAGATCATTGCGTATCTAGA			
	690	700	710	
			
<i>A. flavus</i> 6982 gene(EU016184)	TGCTCAGG~GCTACTTGCCCCCTAAGAAGGAGTAG			
<i>Af6982conins</i> gene(EU016185)	TGCTCAGG~GCTACTTGCCCCCTAAGAAGGAGTAG			
<i>A. nidulans</i> sD gene(YO8866)	TAGCAAGAAGTTGCTTGATGCTTAG			
<i>A. nidulans</i> mRNA(XM_653706)	TAGCAAGAAGTTGCTTGATGCTTAG			
<i>A. fumigatus</i> mRNA(XM_747354.1)	TGAGCAGG~GGTATTTGCCTCCGAAGAAGGAGTAG			
<i>A. clavatus</i> mRNA(XM_001269178)	TGAGCAGG~GTTATCTGCCTCCTAAGAAGGAGTAG			

Figure G-1: Nucleotide sequence alignment of APS kinase from various *Aspergillus* spp. An intron exists in the *A. flavus* gene at bp9-bp86. The GenBank accession number is listed in parentheses following the species name. “.” indicates the same nucleotide as *Af6982*; “~” indicates a gap.

	10	20	30	40
			
<i>A. flavus</i> 6982 gene (EU016186)	ATGCCTGCAAAGATGCCTGATACCTACCCCTCCCACGCTG			
<i>Af6982conins</i> gene (EU016187)	ATGCCTGCAAAGATGCCTGATACCTACCCCTCCCACGCTG			
<i>A. terreus</i> gene (AF123267)	ATGCCCGCGAAGATGCCTGACAACCTACCCCACTGAGTCTG			
<i>A. nidulans</i> gene (X82555)	ATGCCAGCCAAGATGCATTCCAACCTACCCCTCCGATTCTGG			
<i>A. nidulans</i> mRNA (XM_657282)	~~~~~ATGCATTCCAACCTACCCCTCCGATTCTGG			
<i>A. fumigatus</i> mRNA (XM_749859)	ATGCCTGCCAAGTTGCCTTCAAACCTACACCTCTGATGCAG			
<i>A. clavatus</i> mRNA (XM_001270671)	ATGCCTGCGAAGCTGCCTTCCAACCTACCCCTCCGACTCAG			
	50	60	70	80
			
<i>A. flavus</i> 6982 gene (EU016186)	AGG~~~ACTTTGAGTCCAAGGACAATGCCACGGACTCTGG			
<i>Af6982conins</i> gene (EU016187)	AGG~~~ACTTTGAGTCCAAGGACAATGCCACGGACTCTGG			
<i>A. terreus</i> gene (AF123267)	AGG~~~CCTACGATGCCAAAGATACTACCACCGAATCCGG			
<i>A. nidulans</i> gene (X82555)	AGA~~~CTGCGGAACCTAAGAGACTCT~~~ACTGAGTCGGG			
<i>A. nidulans</i> mRNA (XM_657282)	AGA~~~CTGCGGAACCTAAGAGACTCT~~~ACTGAGTCGGG			
<i>A. fumigatus</i> mRNA (XM_749859)	AGGCCGCTGTCGACTCCAGAGATCTTCAGACTGAATCCGG			
<i>A. clavatus</i> mRNA (XM_001270671)	AGCGCGCTATTGATTCCAGAGACCTCCAGACCGACTCTGG			
	90	100	110	120
			
<i>A. flavus</i> 6982 gene (EU016186)	TTATGTTAGTGGTAGCTCGAGTGACGATTATCTCCCCGAG			
<i>Af6982conins</i> gene (EU016187)	TTATGTTAGTGGTAGCTCGAGTGACGATTATCTCCCCGAG			
<i>A. terreus</i> gene (AF123267)	ATATGTCAGTGGTAGCTCCAGCGAGGACTATCTCCCGGAG			
<i>A. nidulans</i> gene (X82555)	CTATGTCAGTGGTGGCTCAAGTGAAGAGTATCTACCAGAG			
<i>A. nidulans</i> mRNA (XM_657282)	CTATGTCAGTGGTGGCTCAAGTGAAGAGTATCTACCAGAG			
<i>A. fumigatus</i> mRNA (XM_749859)	CTATGTCAGTGGGGATTTCGAGTGATGTCTACACTCCAGAG			
<i>A. clavatus</i> mRNA (XM_001270671)	CTATGTCAGTGGAGATTTCGAGTGATGTTTATACGCCAGAG			
	130	140	150	160
			
<i>A. flavus</i> 6982 gene (EU016186)	ATTGTCTTCACGAAACCACATTTGCAGTTTCTCAACAGAC			
<i>Af6982conins</i> gene (EU016187)	ATTGTCTTCACGAAACCACATTTGCAGTTTCTCAACAGAC			
<i>A. terreus</i> gene (AF123267)	ATTGTCTTCACCAAGCCTCATCTCCAGTTCCTCAACCGCC			
<i>A. nidulans</i> gene (X82555)	ATTGTCTTCACCAACCTCATCTTCAGTTCCTCAACCGAC			
<i>A. nidulans</i> mRNA (XM_657282)	ATTGTCTTCACCAACCTCATCTTCAGTTCCTCAACCGAC			
<i>A. fumigatus</i> mRNA (XM_749859)	ATTGTCTTCACCAACCACACCTGCAATTTCTTAACAGGC			
<i>A. clavatus</i> mRNA (XM_001270671)	ATCATTTTCACGAAGACGCACCTGCAGTTTCTGAACAGAC			
	170	180	190	200
			
<i>A. flavus</i> 6982 gene (EU016186)	AGCTGCAGTTCCTTGAGCCTCAAGGTTAGCATAGAGTGGT			
<i>Af6982conins</i> gene (EU016187)	AGCTGCAGTTCCTTGAGCCTCAAGGTTAGCATAGAGTGGT			
<i>A. terreus</i> gene (AF123267)	AGCTGCAGTTCCCTCGAGCCTCAAGGTCGGTCCGCTCGC~TACC			
<i>A. nidulans</i> gene (X82555)	AACTTCAATTCCCTTGAACCCCAAGGTCAGCTC~~~~~TTCC			
<i>A. nidulans</i> mRNA (XM_657282)	AACTTCAATTCCCTTGAACCCCAAG~~~~~TTCC			
<i>A. fumigatus</i> mRNA (XM_749859)	AACTCCAGTTCCTTGAGCCTCAAG~~~~~TTCC			
<i>A. clavatus</i> mRNA (XM_001270671)	AACTCCAGTTCCTTGAGCCTCAAG~~~~~TTCC			

	210	220	230	240
			
<i>A. flavus</i> 6982 gene (EU016186)	CCTCACTCAGTCCACTTGGTAGATGACTAACACCATACTA			
<i>Af6982conins</i> gene (EU016187)	CCTCACTCAGTCCACTTGGTAGATGACTAACACCATACTA			
<i>A. terreus</i> gene (AF123267)	CCTCTTTTTCATCCAAGGGCCAATCACTGACGTGATATTA			
<i>A. nidulans</i> gene (X82555)	ACATTTTTCAGTTCAAGACAACGACGCCTAACCCC~TCTTG			
<i>A. nidulans</i> mRNA (XM_657282)	~~~~~			
<i>A. fumigatus</i> mRNA (XM_749859)	~~~~~			
<i>A. clavatus</i> mRNA (XM_001270671)	~~~~~			

	250	260	270	280
			
<i>A. flavus</i> 6982 gene (EU016186)	TAGATGTCTTGAGATGGTGTGTACATCACTCCCTCACCT			
<i>Af6982conins</i> gene (EU016187)	TAGATGTCTTGAGATGGTGTGTACATCACTCCCTCACCT			
<i>A. terreus</i> gene (AF123267)	~~GATGTCTTGAGATGGTGTGTACATCACTGCCTCATCT			
<i>A. nidulans</i> gene (X82555)	CAGATGTCTCAGATGGTGTGTCACTTCGCTACCTCACCT			
<i>A. nidulans</i> mRNA (XM_657282)	~~~ATGTCTCAGATGGTGTGTCACTTCGCTACCTCACCT			
<i>A. fumigatus</i> mRNA (XM_749859)	~~~AAATCTTGAGATGGTGTATCACATCCCTTCCTCACCT			
<i>A. clavatus</i> mRNA (XM_001270671)	~~~AAATCTTGAGATGGTGCATCACTTCCTTCCTCACCT			

	290	300	310	320
			
<i>A. flavus</i> 6982 gene (EU016186)	TTTCCAGACCACCGCGTTTGGTCTTACTGGACTCGTCACT			
<i>Af6982conins</i> gene (EU016187)	TTTCCAGACCACCGCGTTTGGTCTTACTGGACTCGTCACT			
<i>A. terreus</i> gene (AF123267)	CTTTCAGACCACCGCCTTCGGTCTGACCGGCCTGGTCACG			
<i>A. nidulans</i> gene (X82555)	GTATCAGACCACCGCCTTGGTCTTACTGGTCTTGTGATC			
<i>A. nidulans</i> mRNA (XM_657282)	GTATCAGACCACCGCCTTGGTCTTACTGGTCTTGTGATC			
<i>A. fumigatus</i> mRNA (XM_749859)	CTTCCAGACCACCTGCGTTTGGTTTGAAGTGGACTTGTACAC			
<i>A. clavatus</i> mRNA (XM_001270671)	CTTCCAGACCACCGCCTTGGGCTGACTGGTCTGGTCAC			

	330	340	350	360
			
<i>A. flavus</i> 6982 gene (EU016186)	CTTGATATGCTTTCCAAGCTGGAGGTCCCTCGCCCTCAGA			
<i>Af6982conins</i> gene (EU016187)	CTTGATATGCTTTCCAAGCTGGAGGTCCCTCGCCCTCAGA			
<i>A. terreus</i> gene (AF123267)	CTGGACATGCTCTCCAAGCTGGACGTGCCCCGCCCCAGG			
<i>A. nidulans</i> gene (X82555)	ATGGATATGCTTTCCAAACTGTCCATCCCTCGCCCTCAAA			
<i>A. nidulans</i> mRNA (XM_657282)	ATGGATATGCTTTCCAAACTGTCCATCCCTCGCCCTCAAA			
<i>A. fumigatus</i> mRNA (XM_749859)	CTGGACATGCTTTCCAAGCTTCAAGTGCCCCGCCCCACAGA			
<i>A. clavatus</i> mRNA (XM_001270671)	CTCGACATGCTCTCCAAGCTCAAGGTGCCTCGCCCCACAGA			

	370	380	390	400
			
<i>A. flavus</i> 6982 gene (EU016186)	TGGTCGACCTCATCTTCCTGGACACTCTTCATCACTTCTC			
<i>Af6982conins</i> gene (EU016187)	TGGTCGACCTCATCTTCCTGGACACTCTTCATCACTTCTC			
<i>A. terreus</i> gene (AF123267)	TGGTCGACCTGGTCTTCCTCGACACCTCCACCACTTCAA			
<i>A. nidulans</i> gene (X82555)	TGGTCAACCTCATCTTCCTCGACACTCTGCACCACTTCCC			
<i>A. nidulans</i> mRNA (XM_657282)	TGGTCAACCTCATCTTCCTCGACACTCTGCACCACTTCCC			
<i>A. fumigatus</i> mRNA (XM_749859)	TGGTTGATTTGATCTTCCTTGACACTCTCCATCACTTCCC			
<i>A. clavatus</i> mRNA (XM_001270671)	TGGTCGATCTGATCTTCCTGGACACTCTGCACCACTTCCC			

	410	420	430	440
			
<i>A. flavus</i> 6982 gene (EU016186)	GGAAACCCCTTACTCTGGTCGACAAGATTCGCCAGAAGTAC			
<i>Af</i> 6982conins gene (EU016187)	GGAAACCCCTTACTCTGGTCGACAAGATTCGCCAGAAGTAC			
<i>A. terreus</i> gene (AF123267)	GGAGACCTTGGCTCTGGTTGACAAGGTCCGCCAGCGATAC			
<i>A. nidulans</i> gene (X82555)	CGAGACATTGAAACTTGTGCGACAATGTCCGCAAGAGGTAC			
<i>A. nidulans</i> mRNA (XM_657282)	CGAGACATTGAAACTTGTGCGACAATGTCCGCAAGAGGTAC			
<i>A. fumigatus</i> mRNA (XM_749859)	AGAAACCCCTGGCTCTGGTTGACCGTGTCCGCAAGAGGTAC			
<i>A. clavatus</i> mRNA (XM_001270671)	AGAAACCTTGGCTCTTGTGACCGTGTCCGCAAGAGGTAT			

	450	460	470	480
			
<i>A. flavus</i> 6982 gene (EU016186)	CCCTTGAACAACATCCATGTCTACAAGCCCAAGGGCCTCA			
<i>Af</i> 6982conins gene (EU016187)	CCCTTGAACAACATCCATGTCTACAAGCCCAAGGGCCTCA			
<i>A. terreus</i> gene (AF123267)	CCGAACGTCACCATCCACGTCTACAAGCCCAAGGGCCTGG			
<i>A. nidulans</i> gene (X82555)	CCGCTACAGCACATCCATGTCTACAAGCCCCAGGGTGTTG			
<i>A. nidulans</i> mRNA (XM_657282)	CCGCTACAGCACATCCATGTCTACAAGCCCCAGGGTGTTG			
<i>A. fumigatus</i> mRNA (XM_749859)	CCTCTGAACAACATCCACATCTACAAGCCAGCTGGTATTG			
<i>A. clavatus</i> mRNA (XM_001270671)	CCTCTCAACAACATCCACATCTACAAGCCTGCCGCGCTCG			

	490	500	510	520
			
<i>A. flavus</i> 6982 gene (EU016186)	ACTCTGAAGAAGAGTTTGCCAAGAAGTACGGTGCCCGTCT			
<i>Af</i> 6982conins gene (EU016187)	ACTCTGAAGAAGAGTTTGCCAAGAAGTACGGTGCCCGTCT			
<i>A. terreus</i> gene (AF123267)	AGACGGAAGAGGAATTTGCCAAGAAGTACGGTGCCCGTCT			
<i>A. nidulans</i> gene (X82555)	AGACCGAAGAGGAGTTTGCTAAGAAGCACGGTGAACGCCCT			
<i>A. nidulans</i> mRNA (XM_657282)	AGACCGAAGAGGAGTTTGCTAAGAAGCACGGTGAACGCCCT			
<i>A. fumigatus</i> mRNA (XM_749859)	AAACTGCGGAAGAGTTTTCGAAGAAGTACGGTGCCAAGCT			
<i>A. clavatus</i> mRNA (XM_001270671)	AGAGTGAAGAGGAGTTCTCCAAGAAGTATGGAGCCAAGTT			

	530	540	550	560
			
<i>A. flavus</i> 6982 gene (EU016186)	ATGGGAACGGGATGATCAGCTCTACGACTGGGCGGCAAG			
<i>Af</i> 6982conins gene (EU016187)	ATGGGAACGGGATGATCAGCTCTACGACTGGGCGGCAAG			
<i>A. terreus</i> gene (AF123267)	CTGGGAGCGGGATGACCAGTTCTACGACTGGGTGGCCAAG			
<i>A. nidulans</i> gene (X82555)	GTGGGAAAAGGATGACCAGCTGTACGACTGGATTGCCAAG			
<i>A. nidulans</i> mRNA (XM_657282)	GTGGGAAAAGGATGACCAGCTGTACGACTGGATTGCCAAG			
<i>A. fumigatus</i> mRNA (XM_749859)	CTGGGAGACAGACGATCAGTTCTACGACTGGGTGGCCAAG			
<i>A. clavatus</i> mRNA (XM_001270671)	GTGGGAGACTGATGACCAGTTCTACGACTGGGTGGCCAAG			

	570	580	590	600
			
<i>A. flavus</i> 6982 gene (EU016186)	GTAGAGCCCGCCAGCGTGCCTACCGTGAACCTGAACGTTT			
<i>Af</i> 6982conins gene (EU016187)	GTAGAGCCCGCCAGCGTGCCTACCGTGAACCTGAACGTTT			
<i>A. terreus</i> gene (AF123267)	GTCGAGCCGGCTCAGCGGGCCTACCGTGAACCTCAACGTTT			
<i>A. nidulans</i> gene (X82555)	GTCGAGCCTGCTCAACGTGCCTACCGTGAGCTCAACGTTT			
<i>A. nidulans</i> mRNA (XM_657282)	GTCGAGCCTGCTCAACGTGCCTACCGTGAGCTCAACGTTT			
<i>A. fumigatus</i> mRNA (XM_749859)	GTCGAACCGGCCAGCGTGCCTATCGTGAACCTCAACGTTT			
<i>A. clavatus</i> mRNA (XM_001270671)	GTTGAACCAGCCCAGCGCGCCTACCGTGAACCTCAACGTTT			

	610	620	630	640
			
<i>A. flavus</i> 6982 gene (EU016186)	ATGCTGTCCTCACC	GGCCGTCGCCG	TAGCCAGGGTGGTAA	
<i>Af6982conins</i> gene (EU016187)	ATGCTGTCCTCACC	GGCCGTCGCCG	TAGCCAGGGTGGTAA	
<i>A. terreus</i> gene (AF123267)	ACGCCGTGCTGACT	TGGCCGTCGTC	GCAGCCAGGGTGGCAA	
<i>A. nidulans</i> gene (X82555)	ACGCAGTCCTCACC	GGACGCCGCCG	CAGCCAAGGAGGCAA	
<i>A. nidulans</i> mRNA (XM_657282)	ACGCAGTCCTCACC	GGACGCCGCCG	CAGCCAAGGAGGCAA	
<i>A. fumigatus</i> mRNA (XM_749859)	ATGCTGTCCTCACT	TGGACGCCGCC	CAGTCAGGGTGGCAA	
<i>A. clavatus</i> mRNA (XM_001270671)	ATGCTGTCCTCACT	TGGACGCCGCC	CAGTCAGGGTGGCAA	

	650	660	670	680
			
<i>A. flavus</i> 6982 gene (EU016186)	GCGTGGTGACCTTG	ACATCATCGAGG	TGACGAGGCTGGT	
<i>Af6982conins</i> gene (EU016187)	GCGTGGTGACCTTG	ACATCATCGAGG	TGACGAGGCTGGT	
<i>A. terreus</i> gene (AF123267)	GCGGGGAGACCTCG	ACATCATCGAAG	TGCACGAGGCTGGT	
<i>A. nidulans</i> gene (X82555)	GCGTGGAGACCTGG	ACATTATTGAGG	TGGACGAAGCCGGC	
<i>A. nidulans</i> mRNA (XM_657282)	GCGTGGAGACCTGG	ACATTATTGAGG	TGGACGAAGCCGGC	
<i>A. fumigatus</i> mRNA (XM_749859)	GCGTGGCGATCTCG	ATGTGATCGAGG	TGCACGAGGCCGGC	
<i>A. clavatus</i> mRNA (XM_001270671)	GCGTGGTGATCTCG	ATGTGATTGAGG	TGCACGAGGCTGGT	

	690	700	710	720
			
<i>A. flavus</i> 6982 gene (EU016186)	CTTATCAAGATCAAC	CCTCTGGCCAAC	TGGACTTTTCGATC	
<i>Af6982conins</i> gene (EU016187)	CTTATCAAGATCAAC	CCTCTGGCCAAC	TGGACTTTTCGATC	
<i>A. terreus</i> gene (AF123267)	CTCATCAAGATCAAC	CCGCTGGCTAAC	TGGACCTTTGAGC	
<i>A. nidulans</i> gene (X82555)	CTCATTAAGATCAAC	CCTCTCGCCAAC	TGGACCTTTGACC	
<i>A. nidulans</i> mRNA (XM_657282)	CTCATTAAGATCAAC	CCTCTCGCCAAC	TGGACCTTTGACC	
<i>A. fumigatus</i> mRNA (XM_749859)	CTCATCAAGGTCAAC	CCTCTGGCCAAC	TGGACTTTTCGAAC	
<i>A. clavatus</i> mRNA (XM_001270671)	CTGATCAAGGTCAAC	CCTCTAGCCAAC	TGGAACCTTTGAGC	

	730	740	750	760
			
<i>A. flavus</i> 6982 gene (EU016186)	AAGTCAAGCAGTAC	ATCAAAGACAAC	GATGTGCCCTACAA	
<i>Af6982conins</i> gene (EU016187)	AAGTCAAGCAGTAC	ATCAAAGACAAC	GATGTGCCCTACAA	
<i>A. terreus</i> gene (AF123267)	AGGTCAAGGCCTAC	ATCAAGGAGAAC	ACGTGCCCTACAA	
<i>A. nidulans</i> gene (X82555)	AGGTCAAGCAGTAT	TGTCAAGGAGAA	TGATATCCCCTACAA	
<i>A. nidulans</i> mRNA (XM_657282)	AGGTCAAGCAGTAT	TGTCAAGGAGAA	TGATATCCCCTACAA	
<i>A. fumigatus</i> mRNA (XM_749859)	AAGTGAAACAATA	CATCAAGGAGAA	CGATGTGCCCTACAA	
<i>A. clavatus</i> mRNA (XM_001270671)	AAGTTAAGCAGTAT	TGTCCAGGAGAA	CGATGTTCCTACAA	

	770	780	790	800
			
<i>A. flavus</i> 6982 gene (EU016186)	TGAGCTGCTTGACC	GCGGCTACAAG	AGCATTGGTGACTAC	
<i>Af6982conins</i> gene (EU016187)	TGAGCTGCTTGACC	GCGGCTACAAG	AGCATTGGTGACTAC	
<i>A. terreus</i> gene (AF123267)	CGAGCTCCTTGACC	GCGGCTACAAG	AGCATCGGGGACTAC	
<i>A. nidulans</i> gene (X82555)	CGAGTTACTCGACA	AGGGCTACAAG	AGCGTTGGTGACTAT	
<i>A. nidulans</i> mRNA (XM_657282)	CGAGTTACTCGACA	AGGGCTACAAG	AGCGTTGGTGACTAT	
<i>A. fumigatus</i> mRNA (XM_749859)	CGAACTGCTGGACC	GCGGCTACAAG	AGCATCGGTGACTGG	
<i>A. clavatus</i> mRNA (XM_001270671)	TGAACTGCTGGACC	GCGGCTACAAG	AGCATCGGTGACTGG	

	810	820	830	840
			
<i>A. flavus</i> 6982 gene (EU016186)	CACTCTACCCAACCCGTC	CAAGGAAAACGAGGAT	GAACGGT	
<i>Af</i> 6982conins gene (EU016187)	CACCTCTACCCAACCCGTC	CAAGGAAAACGAGGAT	GAACGGT	
<i>A. terreus</i> gene (AF123267)	CACTCCACCCAGCCCGTC	CAGCGAGAACGAGGAT	GAACGGT	
<i>A. nidulans</i> gene (X82555)	CACCTCTACATCCCCCGTC	CAAGGAGAACGAAGACGAACGAT		
<i>A. nidulans</i> mRNA (XM_657282)	CACCTCTACATCCCCCGTC	CAAGGAGAACGAAGACGAACGAT		
<i>A. fumigatus</i> mRNA (XM_749859)	CACCTCCACACAGCCTGT	CAAGGAGAATGAGGACGAGCGAT		
<i>A. clavatus</i> mRNA (XM_001270671)	CACTCCACCCAGCCCGTC	CAAGGAGAACGAAGATGAGAGAT		
	850	860	870	880
			
<i>A. flavus</i> 6982 gene (EU016186)	CCGGTCGCTGGAAGGGCCAAGCCAAGACCGAATGTGGCAT			
<i>Af</i> 6982conins gene (EU016187)	CCGGTCGCTGGAAGGGCCAAGCCAAGACCGAATGTGGCAT			
<i>A. terreus</i> gene (AF123267)	CGGGCCGCTGGAAGGGCCAGGAGAAGACCGAGTGTGGCAT			
<i>A. nidulans</i> gene (X82555)	CAGGCCGCTGGAAGGGCCAGGCCGAAAACCGAGTGTGGTAT			
<i>A. nidulans</i> mRNA (XM_657282)	CAGGCCGCTGGAAGGGCCAGGCCGAAAACCGAGTGTGGTAT			
<i>A. fumigatus</i> mRNA (XM_749859)	CTGGTCGCTGGAAGGGCCAGGAGAAGACCGAGTGTGGCAT			
<i>A. clavatus</i> mRNA (XM_001270671)	CCGGTCGCTGGAAGGGTCAGGCCAAGACCGAGTGTGGCAT			
	890	900	910	920
			
<i>A. flavus</i> 6982 gene (EU016186)	CCACAACCCTCGCTCCAAGTATGCCCAATACCTGATGGAG			
<i>Af</i> 6982conins gene (EU016187)	CCACAACCCTCGCTCCAAGTATGCCCAATACCTGATGGAG			
<i>A. terreus</i> gene (AF123267)	CCACAACCCTCGCTCCAAGTACGCCCAGTACCTGCTGGAC			
<i>A. nidulans</i> gene (X82555)	CCACAACCCTCGGTCAAAGTACGCCCAGTACTTGATGGAT			
<i>A. nidulans</i> mRNA (XM_657282)	CCACAACCCTCGGTCAAAGTACGCCCAGTACTTGATGGAT			
<i>A. fumigatus</i> mRNA (XM_749859)	CCACAACCCCGCTTCCAAGTACGCCCAGTACCTGATGGAG			
<i>A. clavatus</i> mRNA (XM_001270671)	CCACAACCCTCGCTCCAAGTACGCCCATATCTGATGGAA			
	930	940	950	960
			
<i>A. flavus</i> 6982 gene (EU016186)	CTTGAGCGCAAGCGGCAGGAGGAGGCCCTGTCTCAGGCGC			
<i>Af</i> 6982conins gene (EU016187)	CTTGAGCGCAAGCGGCAGGAGGAGGCCCTGTCTCAGGCGC			
<i>A. terreus</i> gene (AF123267)	ATGGAGCGCAA~CGGCAGGAGGAGGCTCTCTACAGGCGT			
<i>A. nidulans</i> gene (X82555)	ATGGAAC~CAAGCGACAGGAGGAAG~			
<i>A. nidulans</i> mRNA (XM_657282)	ATGGAACGCAAGCGACAGGAGGAAGCTTTGTCTCAAGCGT			
<i>A. fumigatus</i> mRNA (XM_749859)	CTGGAGCGTAAGAGACAGGAGGAAGCTCTATCACAGGCAC			
<i>A. clavatus</i> mRNA (XM_001270671)	TTGGAGCGTCAGCGGCAGGAGGAGGCTTTATCGCAGGCGC			
	970	980	990	
			
<i>A. flavus</i> 6982 gene (EU016186)	TGCAGAACCAAATGACCACTGCTCAATGA			
<i>Af</i> 6982conins gene (EU016187)	TGCAGAACCAAATGACCACTGCTCAATGA			
<i>A. terreus</i> gene (AF123267)	TGA			
<i>A. nidulans</i> gene (X82555)	~~~~~CTTACCACCGCTTAATAA			
<i>A. nidulans</i> mRNA (XM_657282)	TGCAGAACAAGCTTACCACCGCTTAA			
<i>A. fumigatus</i> mRNA (XM_749859)	TTCAAAACCAGCTCACCACCTGCGTAG			
<i>A. clavatus</i> mRNA (XM_001270671)	TGCAGACTCAGCTCGCCGTCGGTTCGGAGTAG			

Figure G-2: Nucleotide sequence alignment of PAPS reductase from various *Aspergillus* spp. An intron exists in the *A. flavus* gene at bp185-bp243. The GenBank accession number is listed in parentheses following the species name. “.” indicates the same nucleotide as *Af*6982; “~” indicates a gap.

Appendix H

Appendix H shows the growth curves of *Af6982* and *Af6982con^{ins}* on 1% cuticle agar and SGM agar media assessed using average hyphal length.

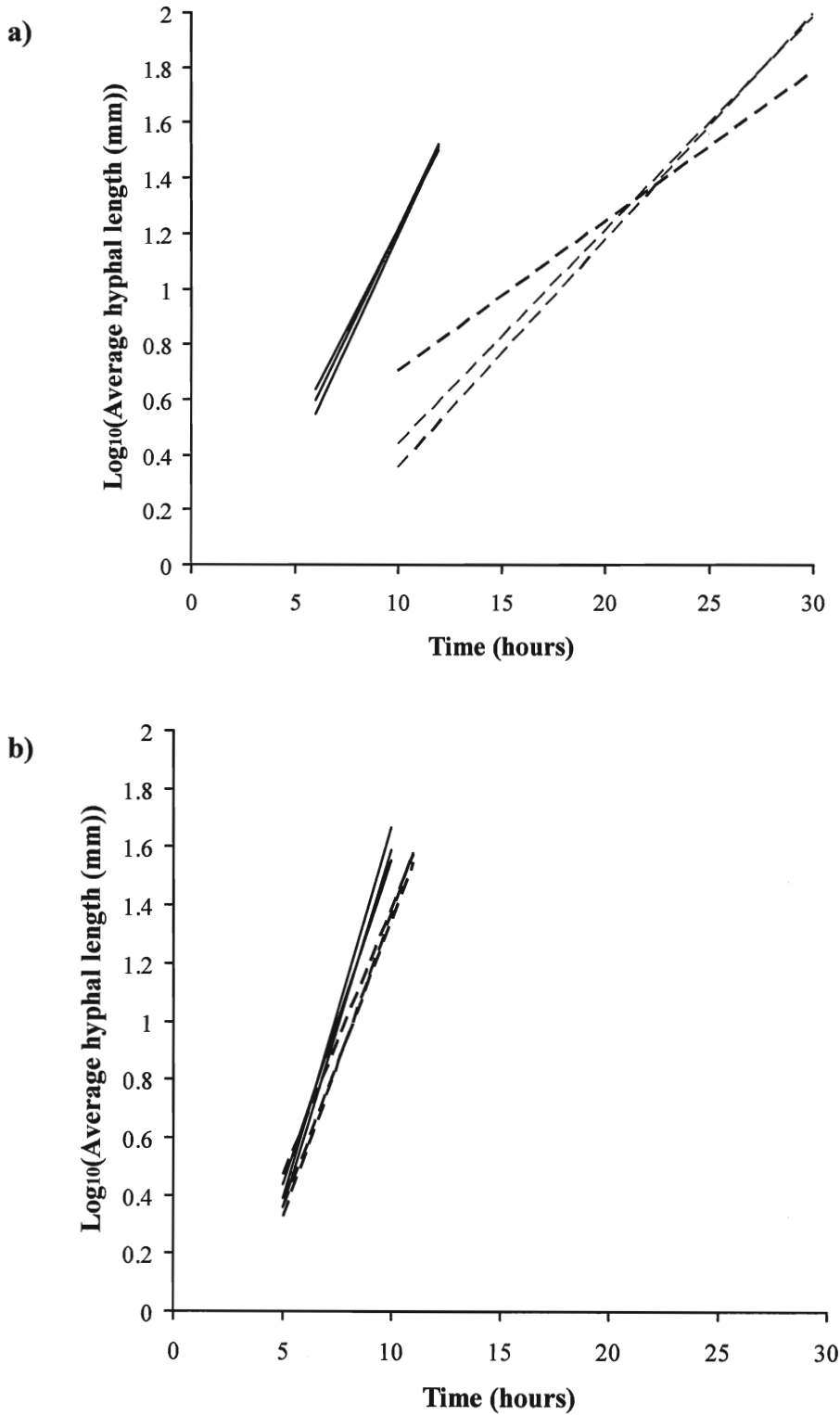


Figure H-1: Growth curves of *Af6982* (solid lines) and *Af6982con^{ins}* (hatched lines) on (a) 1% cuticle agar and (b) SGM agar incubated at 30°C. Curves were constructed by calculating log_{10} of the average hyphal length (mm) at various time intervals.

Appendix I

Appendix I provides an example of hyphae of a GFP-expressing *A. flavus* transformant viewed and photographed using a Leitz Diaplan microscope with a fluorescein filter set (488nm), representative of all putative and confirmed transformants.

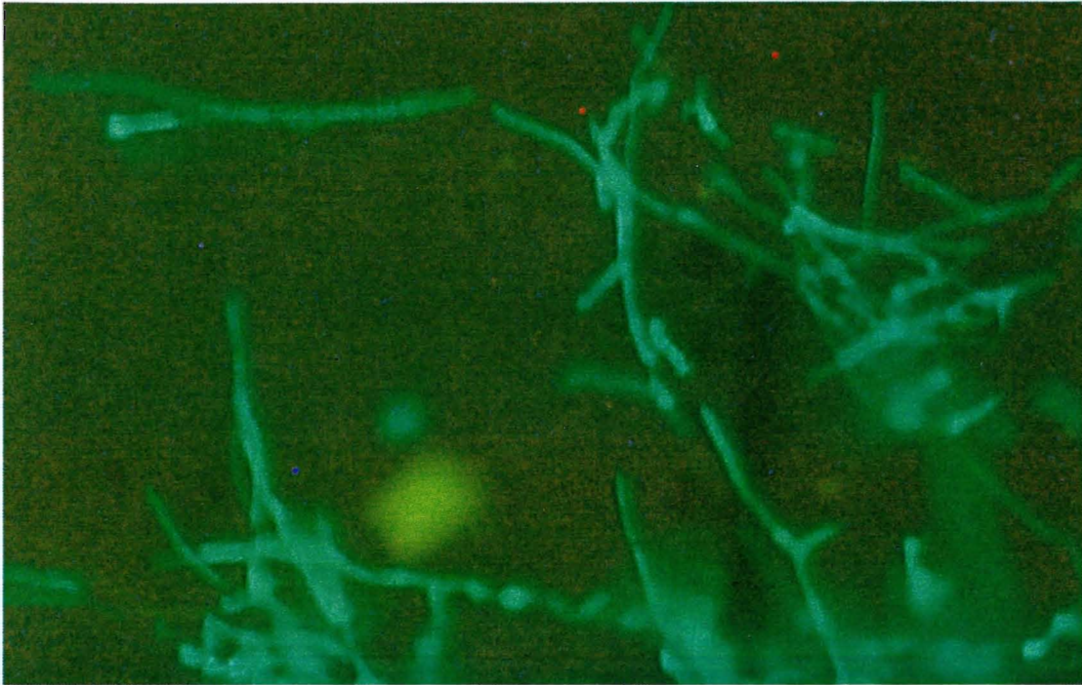


Figure I-1: Microscopic examination of hyphae of a strain of *A. flavus* putatively transformed with pPTRI-GFP-prlA, viewed and photographed using a Leitz Diaplan microscope with a fluorescein filter set (488nm).