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Strain improvement of fungal insecticides for controlling insect pests and vector-borne diseases

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Insect pathogenic fungi play an important natural role in controlling insect pests. However, few have been successfully commercialized due to low virulence and sensitivity to abiotic stresses that produce inconsistent results in field applications. These limitations are inherent in most naturally occurring biological control agents but development of recombinant DNA techniques has made it possible to significantly improve the insecticidal efficacy of fungi and their tolerance to adverse conditions, including UV. These advances have been achieved by combining new knowledge derived from basic studies of the molecular biology of these pathogens, technical developments that enable very precise regulation of gene expression, and genes encoding insecticidal proteins from other organisms, particularly spiders and scorpions. Recent coverage of genomes is helping determine the identity, origin, and evolution of traits needed for diverse lifestyles and host switching. In future, such knowledge combined with the precision and malleability of molecular techniques will allow design of multiple pathogens with different strategies and host ranges to be used for different ecosystems, and that will avoid the possibility of the host developing resistance. With increasing public concern over the continued use of synthetic chemical insecticides, these new types of biological insecticides offer a range of environmental-friendly options for cost-effective control of insect pests.

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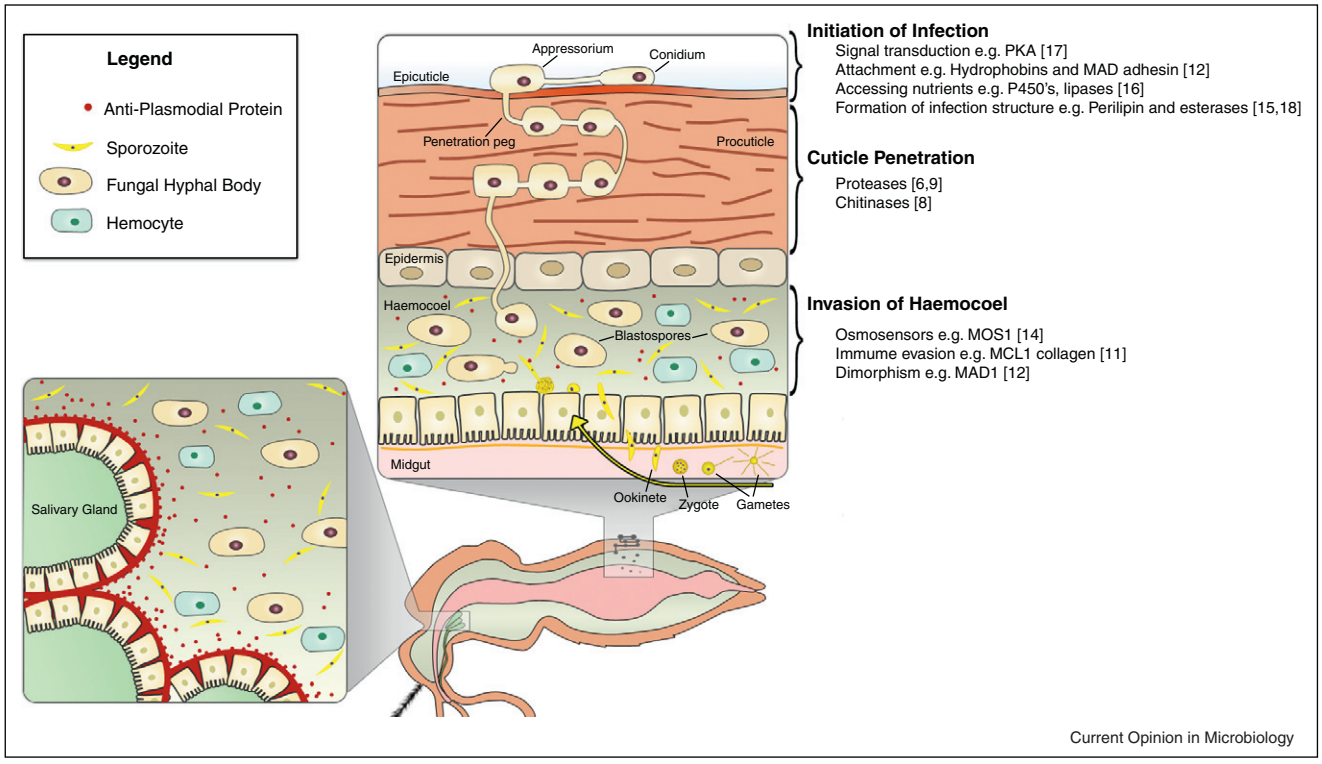
Introduction

While many of the approximately 1000 known species of entomopathogenic fungi have narrow host ranges, collectively they target most if not all insect species including sucking insects, and the many coleopteran and orthopteran pests, among others, which have few known viral or bacterial diseases [1]. Fungi can target sucking insects such as mosquitoes because unlike bacteria and viruses they do not require ingestion by the host. Instead, these fungi infect by direct penetration of the cuticle. Following penetration the fungus propagates in the insect hemocoel. Upon the death of the insect host, hyphae reemerge to cover the cadaver and produce massive numbers of conidia to infect new hosts (Figure 1). Industrial production of *Metarhizium* spp. is highly automated and the price of commercialized *Metarhizium acridum* for locust control in Africa, Australia, and China works out at US$20/ha for 50 g/ha, which is similar to the price of conventional chemical insecticides [2]. However, fungal pathogens have a small market share because of inconsistencies in performance and low virulence (slow kill and high inoculum load) compared to the chemicals with which they compete. Low efficacy could be inbuilt because an evolutionary balance may have developed between microorganisms and their hosts so that quick kill, even at high doses, is not adaptive for the pathogen, in which case cost-effective biocontrol will require genetic modification of the fungus [3]. Better understanding of fungal pathogenesis in insects and the availability of efficient tools for genetic manipulation is alleviating efficacy limitations by allowing construction of transgenic strains with improved ability to kill insects, tolerate adverse conditions and tackle vector-borne diseases. With increasing public concern over the continued use of synthetic chemical insecticides, these new types of biological insecticides offer a range of environmental-friendly options for cost-effective control of insect pests [4].

Genetic engineering to improve virulence

Genetic engineering to improve virulence has focused on reducing both lethal spore dosage and time to kill (Table 1). Reducing spore dosage improves infection rates allowing control to be achieved with less product. It also increases effective persistence of the biopesticide because as spores decay there is a greater probability that an insect will come into contact with enough propagules of the genetically engineered fungus to exceed the inoculum threshold [5]. Most studies to date have exploited the insect pathogenic fungi themselves as a resource of genes for strain improvement (Table 1). In the first example of a recombinant fungal pathogen with enhanced virulence, additional copies of the gene encoding the regulated cuticle degrading protease Pr1 were inserted into the genome of *Metarhizium anisopliae* and constitutively overexpressed. The resultant strain showed a 25% mean reduction in survival time (LT_{50}) toward *Manduca sexta* as

Figure 1



Infection pathway for *M. anisopliae* (upper zoom) and using genetically modified fungi to block malaria transmission (left zoom). Infection is initiated by a conidium that lands on the cuticle and produces a large sticky holdfast (appressorium) that generates mechanical pressure and cuticle degrading enzymes. Unlike bacteria and viruses that need to be ingested to cause disease, fungi kill insects by direct penetration of the cuticle followed by multiplication in the hemocoel as a budding yeast like phase called blastospores. During infection processes the fungus adapts to several distinct environments including the hydrophobic wax-rich epicuticle, the protein–chitin procuticle, and the cellular hypodermis before reaching the solute-rich hemolymph. Our studies have shown that each step requires differential expression of hundreds of genes including those for signal transduction, cuticle degrading enzymes, stress responses, immune evasion, and cell wall reorganization. The brackets point to references for some key genes for each step in the infection process. The hemolymph dependent promoter of the immune evasion gene *Mcl*1 is being used to drive transgene expression by blastopores. A mosquito becomes infected with malaria when it takes a blood meal from an infected human. Once ingested, the parasite will further differentiate into male or female gametes which then fuse in the mosquito's gut. This produces an ookinete that penetrates the gut lining and produces an oocyst in the gut wall. When the oocyst ruptures, it releases sporozoites that migrate through the mosquito's body to the salivary glands, where they are then ready to infect a new human host. The fungus and the plasmodium thus coinhabit the hemolymph, and the fungus can be used to produce antimalarial proteins that within a couple of days inactivate sporozoites and/or block their invasion into salivary glands [37••]. The fungus can also be used to express insect-specific toxins that attack the host's nervous system [25••].

compared to the parent wild-type strain [6•]. Importantly, a Pr1 overexpressing strain of *M. anisopliae* was used in the first EPA approved field trial of a transgenic fungal pathogen, thus breaching regulatory barriers and paving the way for future trials [7]. Insect cuticle also contains chitin as a structural component. Constitutive overproduction of *Beauveria bassiana*'s chitinase CHIT1 improved virulence by 23% [8]. Overexpressing both Pr1-like protease BbCDPE1 and chitinase Bbchit1 in *B. bassiana* decreased the spore dose needed to kill by 67% [9].

The complete sequencing of *M. acridum* and *Metarhizium robertsii* has been completed and is helping determine the identity, origin, and evolution of traits needed for diverse lifestyles and host switching [10]. Success in developing transgenic organisms will benefit from knowledge of the signal transduction pathways that regulate pathogenesis, particularly host range, and the availability of a wide range of suitable genes that can be used to increase virulence. The range of genes is likely to be enormous as adhesins, species-specific toxin-encoding genes and systems for evading host immunity have probably evolved independently in many insect pathogens (Figure 1) [10–16]. We have already identified signal transduction pathways including protein kinase A (MaPka1), that are master regulators of insect infection processes [17], and begun to identify the downstream regulators of differentiation which can be used to target fungi to specific hosts, or change host range. An esterase involved in mobilizing internal nutrients in the broad host range *M. robertsii* was used to transform the locust-specific *M. acridum* into a

Table 1

Genes and metabolic pathways that have been used to improve fungal virulence and tolerance to adverse conditions.

Aim	Type	Source	Function
Improve virulence			
Genes derived from insect pathogens			
Pr1A	Subtilisin-like protease	*Metarhizium anisopliae*	Degrading insect cuticle
Bbcdep-1	Subtilisin-like protease	*Beauveria bassiana*	Degrading insect cuticle
Bbchit1	Chitinase	*Beauveria bassiana*	Degrading insect cuticle
ViaP3	Vegetative insecticidal proteins	*Bacillus thuringiensis*	Lyse midgut epithelium cells, forming pores on the cell membrane
Gene derived from a scorpion			
AaIT	Sodium channel blocker	*Androctonus australis*	Selectively modify the gating mechanism of insect's sodium channel
Improve tolerance to adverse environmental conditions			
Try	Tryosinase	*Aspergillus fumigatus*	Production of pigments
BbSOD1	*Superoxide dismutase*	*Beauveria bassiana*	Detoxify reactive oxygen species
DHN-melanin synthesis pathway	Three genes	*Alternaria alternate*	Production of DHN-melanin

pathogen of caterpillars [18]. It is also possible to mix and match virulence genes from insect pathogenic viruses and bacteria with those from fungi to create novel combinations of insect specificity and virulence. Expressing a gut active toxin (Vip3A) from *Bacillus thuringiensis* (Bt) did not increase the infectivity of *B. bassiana* spores, but in contrast to the wild type, the transformants were also lethal following ingestion [19]. Bt toxins expressed by *B. bassiana* may also be more environmentally stable as, for example, when expressed by *Pseudomonas* and *Rhizobium* bacteria [20,21]. Finally, microbial pathogenicity genes have also been combined with components of arthropod genes to produce new types of anti-insect proteins. For example, *B. bassiana* chitinase Bbchit1 lacks chitin-binding domains and is twofold more effective at degrading insect cuticular chitin when combined with an insect chitin-binding domain. Expression of wild-type Bbchit1 or the hybrid chitinase improved the virulence of *B. bassiana* by 18% and 23%, respectively [22].

In spite of their potential for strain improvement, microbial genes have not yet produced the leap to hypervirulence necessary for a breakthrough product. Arthropod neuropeptides are a particularly attractive alternative to microbial toxins as they offer a high degree of biological activity, and rapidly degrade in the environment providing environmental safety [23]. Over one million peptide toxins have been isolated from arachnids and scorpions, but their use for pest control has been limited since they are not toxic *per os*, and require a means of delivery into the circulatory system. We combined some of these toxins with the natural ability of insect pathogenic fungi to penetrate into insects. We initially tested AaIT (a sodium channel blocker) because it is well studied and very potent and so would provide a benchmark for efficacy [24]. The modified *M. anisopliae* expressing AaIT under the control of a hemolymph-specific promoter (to prevent expression outside an insect) achieved the same mortality rates in tobacco hornworm (*M. sexta*) at 22-fold lower spore doses than the wild type [25••]. Similar results were obtained with mosquitoes (LC_{50} reduced ninefold) and Broca (coffee berry borer beetle; LC_{50} reduced 16-fold) [26]. Toxins from funnel web spiders have proven to be even more potent than AaIT against some insects (Fang, St. Leger, unpublished data).

Improving the tolerance of fungi to abiotic stresses

Abiotic stresses such as UV radiation and high temperature result in fungal insecticides producing inconsistent performances in the field, limiting their use (Fang *et al.*, unpublished data; [27]). Recent studies have shown that these problems can also be solved by genetic engineering. Pigments enhance the survival and competitive abilities of fungi in diverse environments [28]. *M. robertsii* has dark green pigments in spores but it does not produce DHN-melanin that contributes to the tolerance of many other fungi to various abiotic stresses. Tseng *et al.* [29] transferred the DHN-synthesis pathway of *Alternaria alternate* into *M. anisopliae*. Compared to the wild type, the transformant showed a twofold greater tolerance to UV radiation, a 1.3-fold greater tolerance to thermal stress (35 °C) and a 3-fold greater tolerance to low water activity (aw = 97.1%) [29]. Similarly, the tolerance of *B. bassiana* to UV radiation was improved by transforming it with a tyrosinase from *Aspergillus fumigatus* that increased spore pigmentation [30].

UV radiation causes not only DNA damage but also produces reactive oxidative species (ROS) that elevate oxidative stress in cells [31]. Overexpression of a superoxide dismutase (SOD) increased the ability of

Table 2

Antiplasmodium genes that have been used to construct antimalarial fungi.

Gene name	Type	Source	Function
PfNPNA-1	Single-chain antibody	*Human anti-Plasmodium falciparum*	Agglutinates sporozoites
Scorpine	Antimicrobial proteins	*Pandinus imperator*	Inhibits *Plasmodium* development
SM1	Small peptide	*Phage-display library screen*	Blocks attachment of sporozoites to salivary glands

B. bassiana to detoxify ROS, enhancing UV tolerance [32]. The storage carbohydrate trehalose contributes to the thermotolerance of insect pathogenic fungi. Suppressing expression of trehalase in *M. acridum* significantly increased its thermotolerance, but did not alter virulence [33].

Improving the efficacy of fungal insecticides to control vector-borne diseases

Insects and arthropods vector many human, animal, and plant diseases including malaria, bluetongue, and Pierce's disease, and most of these vectors are susceptible to insect pathogenic fungi (Table 2). Laboratory and field studies have demonstrated that insect pathogenic fungi kill adult mosquitoes, albeit slowly [34•,35•]. However, it takes about 14 days for *Plasmodium* to develop from ingested gametocytes to infectious sporozoites (Figure 1). Mosquitoes can be killed in time to block malaria transmission as long as they are infected with fungi at their first or second blood meal. However, the high coverage required for early infection of most mosquitoes in a population may be hard to achieve in the field because of issues such as user resistance [36].

As described above, the virulence of *M. anisopliae* can be increased to a remarkable extent by expressing a scorpion toxin (AaIT) [25••]. However, mosquitoes are notoriously adept at out-evolving control strategies, and a slow speed of kill that enables mosquitoes to achieve part of their lifetime reproductive output could reduce selection pressure for mosquitoes to develop resistance to the biopesticide [5,36]. Fungal strains that greatly reduce mosquito infectiousness could improve disease control without increasing the spread of resistance [5]. To achieve this effect, we produced recombinant strains expressing molecules that target sporozoites as they travel through the hemolymph to the salivary glands (Table 3). Eleven days after a *Plasmodium*-infected blood meal, mosquitoes were treated with *M. anisopliae* expressing salivary gland and midgut peptide 1 (SM1), which blocks attachment of sporozoites to salivary glands; a single-chain antibody that agglutinates sporozoites; or scorpine, which is an antimicrobial toxin. These reduced sporozoite counts by 71%, 85%, and 90%, respectively. Multiple effectors worked synergistically to inhibit sporozoite invasion of salivary glands, and the best combinations (scorpine/SM1:scorpine and scorpine/PfNPNA-1) reduced the sporozoite intensity approximate 98%. Additional benefits included decreased host feeding (and therefore transmission potential) and increased mosquito mortality [37••].

A potential problem with relying on antimalarial effects is that they might in the long run suffer from the evolution of resistant malaria parasites. The single-chain antibody (PfNPNA-1) specifically recognizes the repeat region (Asn-Pro-Asn-Ala) of the *Plasmodium falciparum* surface

Table 3

Disease vectors susceptible to insect pathogenic fungi.

Disease	Vector	Fungi pathogenic to the vector
Vector-borne human diseases		
Mosquito-vectored diseases (Malaria, Dengue fever, among others)	Various mosquitoes	All vector mosquitoes are susceptible to one or more fungal species [44•]
Lyme disease	*Ixodes* spp. ticks	*B. bassiana* [45]
Sleeping sickness	*Glossina* tsetse flies	*M. anisopliae* [46]
Chagas disease	*Triatomine* bugs	*M. anisopliae* and *B. bassiana* [47]
Leishmaniasis	*Lutzomyia* Sunflies	*B. bassiana* [48]
Vector-borne livestock or poultry diseases		
Bluetongue	*Culicoides nubeculosus*	*M. anisopliae*, *B. bassiana*, and *Isaria fumosorosea* [49]
Gall sickness and redwater fever	*Boophilus* spp. and *Stomoxys* spp.	*Metarhizium*, *Beauveria*, and *Lecanicillium* [50]
Heartwater	*Amblyomma* spp.	*Metarhizium* and *Beauveria* [51]
Corridor disease	*Rhipicephalus* spp.	*Metarhizium*, *Beauveria*, and *Lecanicillium* [50]
Vector-borne plant diseases		
Pierce's disease (PD), almond leaf scorch	Glassy-winged sharpshooter	*Metarhizium* [52]

circumsporozoite protein [38], so multiple mutations would be required to achieve resistance. Furthermore, *Metarhizium* is a tractable model for evaluating and delivering transmission blocking proteins and could be used to express multiple transgenes with different modes of action to reduce the probability of emergence of resistance to one mechanism.

Fungal pathogens lend themselves to strategies currently used for delivery of chemical insecticides, for example, being sprayed on indoor surfaces of houses, cotton ceiling hangings, curtains, and bed nets [5], or used in outdoor odor baited traps [39]. In south Asia, human vector mosquitoes feed predominantly on domestic animals and only secondarily on human beings, and applying deltamethrin insecticide to cattle reduced human malaria transmission to the same extent as indoor spraying, but at 80% less cost [40]. *Metarhizium*-based insecticides have been developed and applied to sheep to control mange vector *Psoroptes* mites [41,42], so potentially, transgenic fungal strains could be applied to livestock to simultaneously improve their health and control human malaria.

Fungi can attack almost all known disease vectors (Table 3). Various transgenic fungi could be constructed to express different effector proteins that each attacks one or several vector-borne diseases of humans, animals, and plants. For example, a fungus expressing the antimicrobial scorpion toxin scorpine could control livestock and poultry malaria that are causing significant economic loss [43].

Conclusion and future directions

There are many international crop pest and disease problems that are amenable to biotechnology solutions. Many of these problems could require transgenic technology for which there is only a beginning precedent being set. There is willingness in the regulatory community to take on these issues, but what is most needed are clear and compelling needs, such as malaria control. *M. anisopliae*'s ability to express a functional single-chain antibody fragment is notable because recombinant antibodies provide a vast array of potential antiparasite and anti-arthropod effectors that could target, for example, insect hormone receptors. These would facilitate construction of very effective, highly specific, biopesticides with minimal increased potential for negative environmental impact relative to their parental wild-type strains. The rich arsenal of antiparasite and anti-insect proteins makes it possible that new transgenic strains can be developed that stay one step ahead of the insect or parasite evolving resistance. Given their ease of genetic manipulation, *Metarhizium* and *Beauveria* provide a tractable model system for screening novel effectors or fusion products produced by gene shuffling. The most potent anti-insect or antimicrobial effectors could then be delivered by the fungus, another microbe, and/or in a transgenic insect or plant. Likewise, insect pathogenic fungi could be used to test various metabolic pathways for their ability to enhance tolerance to abiotic stresses.

Given the increasing public acceptance of genetically modified organisms (GMOs), particularly crops expressing *B. thuringiensis* toxins [4], field application of GM insecticidal microbes should have a bright future if care is taken to ensure social acceptance through rigorous risk benefit analysis. GMO projects go through a review process that includes semifield studies in a contained near-natural environment as a prerequisite for an open field release. In our context, this would involve building a malaria sphere that will consist of experimental huts (mimicking traditional housing), sugar sources (plants) for adults and created breeding sites (plastic containers), enclosed in a greenhouse frame with walls of mosquito netting to allow exposure to ambient climate conditions and simulate a natural mosquito habitat. The sphere will allow studies to determine whether the introduced transgenes have the potential to significantly improve the performance of a biocontrol fungus in a disease-endemic setting. To a large extent, we think the social and regulatory acceptability of the technology will be resolved by the development of fungi that can significantly reduce malaria occurrence, and concomitantly have no negative environmental impact. We think there is a high likelihood these fungi will be widely accepted by the people who live in areas where their health is impacted.

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