AN OVERVIEW OF *BACILLUS THURINGIENSIS* BIOPESTICIDES

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The leading biological insecticide is based on the common soil bacterium *Bacillus thuringiensis* (Bt). Formulations of these insecticides, containing mixtures of spores and crystals safely control insect pests of vegetables, forestry and vectors of human disease. Despite its high target specificity and environmentally favorable "green" characteristics, the Bt insecticide market commands a small fraction of the global crop protection market.

Between 1980 and the early 1990s there was an extended effort to improve Bt biopesticides. Part of this effort was directed at strain and cry gene isolation yielding over 250 distinct Cry proteins (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/toxins.html). Usually Cry proteins display a narrow spectrum of insecticidal activity against one or more genera in the orders Coleoptera (beetles and weevils), Diptera (flies and mosquitoes), Hymenoptera (wasps and bees), and most importantly Lepidoptera (butterflies and moths), including most of the insect pests of agricultural importance. Some Cry proteins are active against nematodes.

In the early 1990s, a novel encapsulated Cry protein insecticide and recombinant Bt strains and introduced to the U.S. market. Mycogen's MVP product was the first recombinant Bt-based biopesticide to be registered by the U.S. EPA. Ecogen commercialized several recombinant Bt biopesticides. There were also efforts to develop a Bt for control of grass grubs. However, there has been a lack of interest in further developments in the sprayable biopesticide area. The efficacy and economic returns from Bt corn and cotton have restricted the use of novel *cry* genes to transgenic plant applications.

Features of Bt biopesticides limit their use in insect control. In contrast to contact insecticides, Bt insecticides must be ingested by the target insect. The timing of Bt sprays is critical to attaining economic levels of insect control. Usually Bt is applied when early instar larvae are present, as older larvae are more tolerant. Bt sprays persist only a few days on the leaf surface. The chemistry of the leaf surface, proteinases and sunlight contribute to the degradation of Cry proteins. It is rare for a Bt insecticide to have greater efficacy than the best available chemical control. Hence, Bt adoption suffers at the hand of more efficacious chemical insecticides.

Bt biopesticides have inherent advantages in certain pest control applications. They are used as a resistance management tools in insect control. Due to their distinct mode-of-action, they are alternated or combined with chemical pesticides. Bt is especially suited for specialty or 'high value crops.' The tightening of registration procedures for new chemical pesticides has led many of the larger crop protection companies to take the decision not to register products for use on specialty crops. Increased usage of Bt biopesticides will occur as organic markets expand and consumer demand for ecofriendly pest control alternatives in home gardens and treatments for high-value alternative crops.

Impact of Regulatory Decisions on Emerging Pest Control Research

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Pest control research is an enormously broad field. Pests can be segregated into broad biological categories such as rodents, insects, nematodes, weeds, fungi, bacteria. Pests can be categorized by the product or environment in which the pest occurs such as crop pests, stored product pests, forest pests, turf pests, structural pests, vectors of plant or animal (including human) disease, or just nuisances. The battle to control pests is constant; eliminations are rare, but control measures are very effective. Nationwide chains of grocery stores contain both fresh and preserved foods that are nearly devoid of pests or pest damage. Challenges for pest control include recently reported bacterial contamination of bagged spinach, and the increasing presence of *Cimex lectularius*, the common bed bug, in nationwide lodging establishments. Another example of a pest control challenge directly associated with biotechnology is the problem of secondary pests in insect resistant transgenic crops (figure 1).

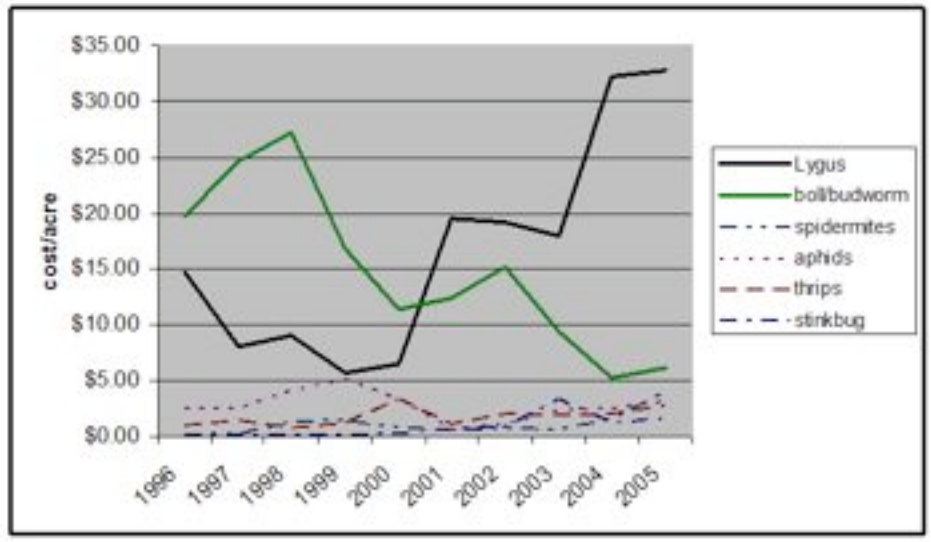

Figure 1. Cotton Insect Losses (Mississippi Summary) data extracted from http://www.msstate.edu/Entomology/Cotton.html (Williams, MR)

Current topics under investigation by pest control research scientists include resistance research, chemistries, molecular genetics, and biological control. These broad topics often overlap.

Resistance research is a critical area of pest control. Resistance to chemical pesticides has been documented for over 50 years. Ongoing research includes detection and monitoring of resistant organisms. Elucidation of pesticide resistance mechanisms at the biochemical and genetic level continues. While some research projects utilize field and in vitro methods, many projects require live pests in the laboratory. Laboratory strains of pesticide resistant pest insects (or fungi, or weeds, etc.) are valuable research assets. Regulations regarding the containment and transfer of biological materials could be viewed as restrictive to scientific progress. A collaborator in another state or country would require a permit from APHIS and/or the state or country, and possibly a facilities inspection or certification.

New chemical compounds and formulations are continually sought and tested for use against pests. Naturally occurring plant compounds that are toxic or repellent to insects, mites, and fungi are reported in pest management and other journals. Synthetic compounds based on known pest toxicology, growth regulation compounds, and chemical communication compounds are reported. Testing these compounds again requires live pests in some sort of containment, and the same constraints mentioned above apply. If a scientist wishes to study effects of a compound on a non-target organism, for example a fungus, another arthropod, a reptile or amphibian, or a mammal, other regulatory hurdles are present.

Molecular genetic studies on pest insects are increasing. Genetic manipulation aimed towards insect control will be described in other presentations, and the progress described is laudable. One must bear in mind however, that sterile insect releases can only work on one species of insect at a time, and there are a multitude of pests, many of which do not fit into the sterile insect paradigm. Molecular genetics and genetic manipulation technology can be powerful tools for gene discovery, and key to the next generation of pest control strategies. Gene discovery projects directed towards control of a wide range of insect pests including human, animal, and plant disease vectors are targeting gene pathways associated with the insect cuticle, insect development, and a wide variety of insect digestive enzymes. Manipulated pest insects on the scale of *D. melanogaster* could speed the path between gene discovery and control methods. Permit and containment requirements for both plant pests and transgenics need to be clear and manageable to facilitate this type of research project.

Biological control is the use of one biological organism to control another, such as releasing beneficial bacteria, fungi, or arthropods to limit pest infestations. Part of the Plant Protection Act states that "biological control is often a desirable, low-risk means of ridding crops and other plants of plant pests and noxious weeds and its use should be facilitated by the […federal agencies] whenever feasible…" Still, regulation has impeded movement of these agents; in recent years there was some concern that providers of biological control agents (BCA's), and biological control research would be regulated out of existance. Regulations prohibiting the movement of biological agents of any sort, instituted in response to the threat of bioterrorism, effectively halted importation of experimental and commercial biological control organisms.

Research on biological control agents and genetic research, especially recombinant genetic research, face difficulties in combining. Many producers of biological control agents (BCA's), and scientist that study them, promote a reputation of environmentalism. The regulatory process, which is very visible by design, could be perceived by those who equate environmentalism as anti-GMO sentiment as a reputation risk.

Part of the APHIS BRS website (http://www.aphis.usda.gov/brs/arthropod_news.html) states that "safe development of agricultural biotechnology products promotes increased public confidence in biotechnology-derived agricultural food and food products." Regulatory agencies also promote safe research; however, instilling public confidence in recombinant DNA research is a bigger challenge.

A RISK HIERARCHY FOR TRANSGENIC VECTOR ARTHROPODS

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Germline transformation has been achieved in an increasing number of arthropods and numerous applications are envisioned. Many of these organisms are vectors of human and animal disease and are being transformed for the purpose of interfering with their capacity to transmit disease. In spite of the intended beneficial endpoint of this effort, descriptions of measures for the safe creation and testing of these animals are necessary. Safe measures for purely laboratory studies have been addressed in the NIH Guidelines for Research Involving Recombinant DNA and the Arthropod Containment Guidelines, but neither of these addresses the conduct of contained field trials planned to occur before 2011.

In an effort to fill this gap in guidelines covering safe field trials of transgenic arthropod vectors, we developed a hierarchical classification of risk factors to consider for evaluating specific projects. This structure recognizes three features of transgenic arthropods that most prominently determine their risk classification: the intrinsic propagation potential of the transgene and its phenotype, the arthropod's sexual fertility status, and the specific phenotype conferred by the transgene.

The realm of commercial and large-scale applications of transgenic organisms consists of stable transgene insertions. Therefore, transgene mobility is a novel characteristic that warrants little concern. By contrast, one planned implementation of transgenic vectors is to inoculate populations with a relatively small number of transgenic organisms and to spread the transgene(s) into non-transgenic populations by various drive mechanisms. Therefore, to assess risk, mobility characteristics must be given special attention. This characteristic will strongly influence the expected variation in the transgene phenotype, and its mobility will produce mutations at insertions sites, both of whose characteristics must be understood clearly. We classify this characteristic – transgene propagation potential – as the first consideration.

The second characteristic, sexual fertility status, affects the probability that the geographic and temporal spread of the transgenic organism and its transgene can be controlled. Releases of sexually sterile transgenic organisms with short life spans and small migration distances will generally be of little long-term consequence. Conversely, release of fertile insects presents the possibility that the transgene may be vertically transmitted to progeny and the concomitant persistence and prevalence of the transgene increases the probability of and unanticipated effects and horizontal transfer.

The third consideration is the phenotype conferred by the transgene. We have assigned these four increasing levels of risk based on how intimately and directly they are expected to affect human health. The lowest level of risk is due to transgenes which contain only a marker. Examples of the application of such transgenes would be as markers for release/recapture and to study gene flow in natural populations. The second level contains transgenes that have an effect on the arthropods life history, for example

killing, sex ratio distortion, or life shortening. Third are transgenes that affect host-pathogen interactions. At the highest level of risk are those transgenes that directly affect human phenotypes. Only one proposal of this type has been described; antigen delivery in mosquito saliva to produce immunity to a pathogen.

TABLE: Relation of the Risk Classification Criteria. The lowest risk organisms are those with characteristics toward the upper left of the table, whereas those with the highest are toward the lower right. Each class is sequentially designated by its propagation potential, reproductive status, and phenotype e.g. 1-S-1 and 2-F-4 are the top left and bottom right classes respectively.

Transgene propagation potential	Vector reproductive status	Phenotype			
		marker only	vector population reduction	disease transmission reduction via non-human route	direct affect on human phenotype
1. Not significant	S-Sterile	1	2	3	4
	F-Fertile	1	2	3	4
2. Significant	S-Sterile	1	2	3	4
	F-Fertile	1	2	3	4

INTERACTIONS BETWEEN FOODBORNE PATHOGENS AND PROTOZOA ISOLATED FROM LETTUCE AND SPINACH

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Previously Brandl et al. (2005) showed that protozoa from moist soil could enhance survival of *Salmonella enterica* by sequestering the bacteria in released food vacuoles (vesicles). Protozoa on fresh produce may similarly protect human pathogens associated with produce. This study examined the ability of protozoa, isolated from produce, to release vesicles containing *Escherichia coli* O157:H7, *S. enterica* (from outbreak sources), and *Listeria monocytogenes* (isolated from mint leaves). Spinach and romaine lettuce (not prepackaged) were rinsed with a saline solution, and the rinse was treated to enrich for protozoan species. Among several protozoan species observed in the rinses, two ciliate species were isolated: *Glaucoma* sp. from the lettuce, and *Colpoda steinii* from the spinach. *Glaucoma* was cultured axenically; *C. steinii* could not be axenized, but was cultured in bacterized cereal leaves medium. In addition to the ciliates, an amoeba sp. was also isolated. A strain of *Tetrahymena pyriformis* (ATCC 30202) originally isolated from moist spinach, and soilborne *Tetrahymena* sp. were also studied; however, the latter one was tested only with *E. coli* O157:H7, because it was tested previously with the other two pathogens (Brandl, et al., 2005). Protozoa were washed and suspended in buffered saline amended with washed GFP- or DsRed-labeled bacterial cells. After 24 h, expelled vesicles were enumerated with a hemacytometer, and ciliates were enumerated microscopically from fixed aliquots. Controls were unfed ciliates. All tests were run twice in duplicate. Vesicles were produced by *Glaucoma* with all bacterial strains, although *Listeria* resulted in the fewest number per ciliate (ave. = 12 and 28 compared with 81 and 220 for *Salmonella*; and 88 and 220 for *E. coli* O157:H7). Vesicle production was also observed with two *Tetrahymena* species. *T. pyriformis* produced vesicles with *E. coli* O157:H7 and *S. enterica* (ave. = 34 and 79 for *E. coli* O157:H7; and 83 and 158 for *S. enterica*) but not with *L. monocytogenes*. *Tetrahymena* sp. produced an average of 30 to 150 vesicles with *E. coli* O157:H7. All vesicles contained intact fluorescing bacteria. No vesicles were observed in controls. Protozoa increased in numbers after they fed on pathogens for 24 h. *T. pyriformis*, the soilborne *Tetrahymena* sp. and *C. steinii* increased to a greater extent than did *Glaucoma* or the amoebae. No change in numbers of *Glaucoma* sp. was observed in controls. However, numbers of *C. steinii*, amoebae and the two *Tetrahymena* species decreased slightly in controls. *C. steinii* and the amoeba species formed cysts, and no entrapment of bacteria inside cysts was observed. Results show that different protozoan species respond differently to foodborne human pathogens; however, two species from produce and one from moist soil can ingest such pathogens and release them in concentrated forms that may protect the bacteria from harmful environmental condition.

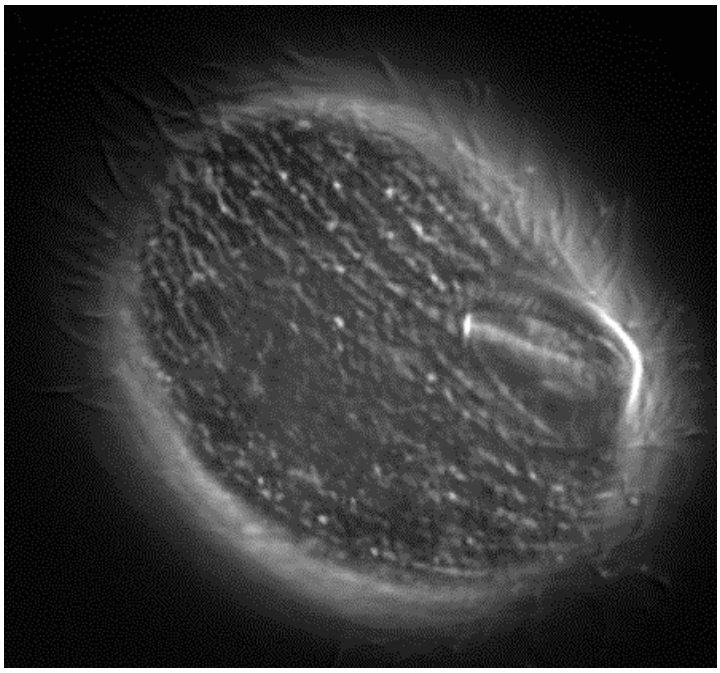

Fig. 1. ***Glaucoma* sp.,** isolated from lettuce.

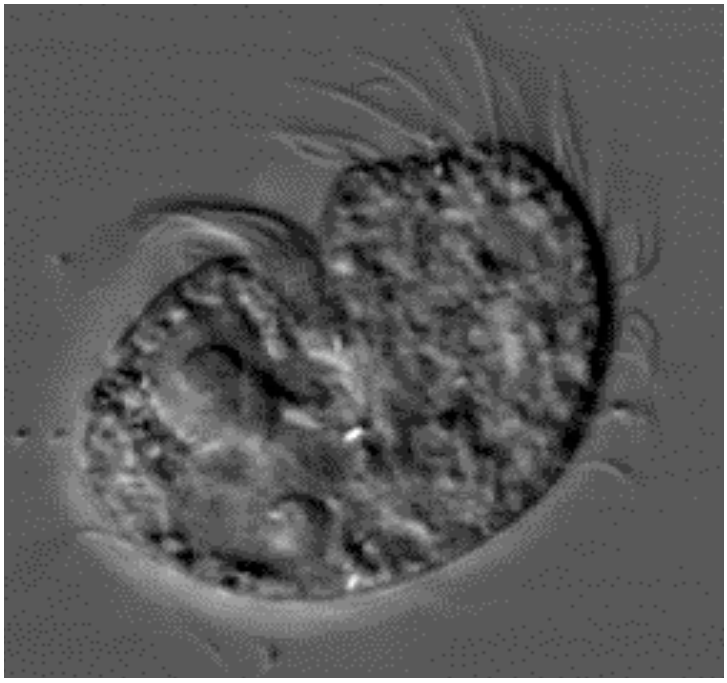

Fig. 2. ***Colpoda steinii***, isolated from spinach.

Reference: Brandl, M.T., B.M. Rosenthal, A.F. Haxo and S.G. Berk. 2005. Enhanced survival of *Salmonella enterica* in vesicles released by a soilborne *Tetrahymena* species. Appl. Environ. Microbiol. **71:**1562-1569.

Use of Transgenic Arthropods in Genetic Control Programmes:

Strain evaluation and risk assessment at the International Atomic Energy Agency

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The IAEA is involved in the assessment and use of modern biotechnology likely to enhance the peaceful use of nuclear energy and to improve the delivery of the Agency's programmes to Member States.

Probably the most suitable pest control strategy for the integration of transgenic technology is the Sterile Insect Technique (SIT), where the presence of the transgene in the environment is limited to the sterile insects that are released. Germline transformation technology is functional in a growing number of arthropods species including species of medical, agricultural and veterinary importance for which SIT is already implemented (fruit flies, tsetse flies, moths) or is under development (mosquitoes). Potential improvements include the development of strains that (1) produce only male insects for sterilization and release and (2) carry a marker that distinguishes them from wild insects, thus increasing the efficiency of these SIT programmes.

However, major technical challenges remain that concern the genotypic and phenotypic stability of the transgenic strains and their ability to express the transgene in a reliable and predictable manner in operational programmes where many millions of insects have to be reared for sterilization and release. A second area of concern is the biological fitness of transgenic strains when they have to compete with individuals in the field. Finally, of much broader significance to the use of transgenic arthropods is the development of an appropriate regulatory framework. Insects that are currently released in SIT programmes are not subject to significant regulatory constraints, but this will change if the released insects are transgenic, even if they are sterile. The release of genetically modified arthropods will require a thorough risk assessment protocol, moving from the laboratory through contained field cages to open field release.

Activities conducted at the IAEA on transgenic insects are designed to better understand their potential and limitations in relation to SIT and to improve decision-making capability in Member States through capacity building in the fields of biosafety and risk assessment.

Technical support

The IAEA conducts adaptive R&D at its laboratories located in Seibersdorf, Austria. Mediterranean fruit fly *Ceratitis capitata* (medfly) SIT programmes in Member States have benefited tremendously through the introduction of genetic sexing strains constructed using conventional breeding techniques. This technology took 15 years to develop and cannot be transferred to other species where SIT programmes are being carried out. However, the potential exists that "generic DNA genetic sexing constructs" can be developed which, through transgenesis, can be introduced into the genome of many different pest species, without the need to develop a separate system for each individual species.

Research on transgenesis essentially focuses on two pest insects: the medfly, a major agricultural pest and the mosquito *Anopheles arabiensis*, one of the three main malaria vectors in Africa. For mosquitoes, it is essential to develop a system to eliminate females, either based on classical breeding techniques or transgenesis, in order to implement an SIT programme. The first germline transformation of *Anopheles arabiensis* was recently achieved at the Entomology Unit using a *piggybac* transformation vector. This represents a significant achievement which opens the

prospect for developing transgenic-based sexing systems potentially allowing the stringent elimination of females required for the release of sterile male mosquitoes.

In all SIT programmes it is essential to mark sterile insects before they are released so as to be able to differentiate them from their wild conspecifics during the evaluation of the programme. This is currently achieved using a fluorescent powder that has major disadvantages for worker health, accuracy and field monitoring costs. Using transgenesis it is possible to mark released insects with a molecular tag that will make recognition unequivocal and easy to implement. Several strains are now being evaluated which carry a molecular tag.

Biosafety and risk assessment of transgenic fruit flies and mosquitoes are being carried out in contained environments to provide credible, independent technical information to organisations in Member States who may wish to consider the use of this technology in their SIT programmes. This information can be used to make science-based decisions as to the risks and benefits associated with the technology. The Agency has considerable expertise and credibility in the field of genetic control of insect pests and the activities on transgenic insects improves considerably the quality of advice for Member States. The evaluation of transgenic strains includes the assessment of transgene stability and consistency of transgene expression under various (mass)-rearing regimes. The insect rearing facility provides an ideal infrastructure to conduct this work. Fitness evaluation of transgenic insect strains in large field cages will be conducted in a fully contained 250m^2 greenhouse currently under construction.

Over the past fifteen years, the Agency has contributed, through the organization of Coordinated Research Projects (CRP) and consultancy meetings, to the development of knowledge in the field of insect transgenesis with direct applications for SIT. The latest CRP specifically addresses the use of transgenic arthropods to improve the effectiveness of the SIT. This CRP initiated in 2004 for a period of 5 years involves more than twenty partners and observers from both developed and developing countries. CRPs thus contribute in building relevant research capacity in developing countries.

Regulatory support

Of much broader significance to the use of transgenic arthropods is the development of a regulatory framework. Current national regulatory processes, including the availability of suitable risk analysis protocols, may be insufficient to address any eventual release of transgenic arthropods. Likewise, the current negative public perception of transgenic technology in general will make the development and use of transgenic arthropods in pest control potentially difficult. Policy making in regard to transgenic arthropods lags far behind that for transgenic plants and micro-organisms, where the latter are now being extensively used in agriculture and human health. However, USDA through APHIS has developed a set of guidelines that were used to issue a permit for the first field cage evaluation of a transgenic plant pest in 2001. More recently, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA, Vienna and the International Plant Protection Convention (IPPC) secretariat, Rome organized a meeting[1] to address the biosafety and regulatory issues described above. This meeting brought together both scientists involved in transgenic technology and experts in the field of risk assessment and regulatory procedures and led to the development of a preliminary set of risk assessment protocols related to the use of transgenic arthropods in agriculture.

The IAEA will continue its efforts in the development of an international regulatory framework including aspects of risk identification, risk assessment and risk management.

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[1] IAEA technical documentation on the Status and risk assessment of the use of transgenic arthropods in plant protection. http://www-pub.iaea.org/MTCD/publications/PDF/te_1483_web.pdf

What can public sector researchers do to facilitate the registration process?

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The transition from research to a marketable product through registration involves science, business, and law. Even within science, many opportunities are missed to assist in the regulatory process. The first thing is to become familiar with registration requirements which include the main areas of 1) Product Characterization and Manufacturing, 2) Toxicology or Health Effects and 3) Non-Target or Environmental Effects. Thinking about science in a regulatory framework involves seeing how the information that is collected through the course of research can be expanded or modified to capture important information pertinent to risk assessment. Risk assessment is a function of hazard (how toxic a substance is) and exposure (frequency, route and length to which some form of contact will occur).

All the applicable data requirements must be satisfied by research studies or data waivers. Toxicology studies can be expensive and take time to organize, conduct and interpret. Data requirements can also be satisfied if there is existing data in the public literature that directly relates to the data requirements. In addition, observational type research that is properly geared toward a data guideline can contribute to a waiver. Therefore the key is recognizing the opportunities for these types of observational studies and seeing how they could be coupled with journal literature relating to hazard or exposure to build a sound scientific argument or waiver.

Negative data(lack of an effect) is often not published. Within a scientists own research, one of the simplest habits to assist registration is to make sure that negative data is recorded and documented so that is available for submission. Simply going back to an old study and saying nothing happened to a plant or animal is not acceptable. It must be recorded at the time it is observed.

The potential routes of exposure and inherent toxicity of the product dictate which studies need to be conducted. For example if a product is to be used only on ornamental crops there is a better chance that acute oral studies may be waived because the treated crop is not eaten. Similarly if a product is restricted to greenhouse use, a number of the environmentally related non-target effects may be waived.

Many biochemicals or organisms may be naturally occurring and routinely found in foods or the environment. While toxicology studies that state actual LD_{50} values are best, acute oral data requirements may also be satisfied by virtue of the fact that the active ingredient is already found in commonly consumed foods. Simply the presence of the same active in foods is not enough. It needs to be related to exposure. By knowing the typical yields of a crop and consumption data the theoretical exposure can be calculated. This can be compared to the known consumption of and content of the active ingredient that is being

registered. The point should be to explain that the theoretical exposure does not constitute a major increase in exposure above that which man is already exposed to. Food scientists and pharmacologists could help in knowing if the active ingredient is also found in foods or medicines and if there are any toxicology studies on the active ingredient.

Dermatologists could help in designing waivers involving dermal toxicity. Many natural products can be found in skin creams, hand cleansers, mouthwash, toothpaste or cosmetics. A caustic substance might not need a dermal toxicity study because its already well documented to be a dermal irritant. Dermal exposure can also be mitigated by protective clothing or by the type of formulation and application equipment involved.

Throughout a research program it important to document the concentrations, lengths of exposure and degrees of exposure involved in routine laboratory and field studies. Although it reflects a limited pool of individuals, affidavits stating a lack of adverse effects from technicians handling the active ingredient can be used as part of the dermal and hypersensitivity justifications.

Avian studies include oral and pulmonary toxicity. Ornithologists may be able to help in their knowledge about birds. This is especially true when it comes to specific endangered bird species. For example if the pesticide is for a specific use that coincides with a particular time of year, the bird may be migratory and not expected to be present during application. Certain birds only live in aquatic environments so potential exposure could be reduced by restricting the label against application to aquatic environments. Birds may have specific diets such as only eating grains or not eating grains which can impact exposure. Sometimes regional geographical restrictions can also limit exposure. Similarly ecologists, ichthyologists and marine biologists should be consulted in regard to wild mammal, freshwater fish, aquatic invertebrates, and marine environments.

Non target insects are one of the more common studies that are actually conducted rather than waived. Within this area, toxicity to honeybees are the most common. Most states have apiculturists that can assist in setting up observational type studies. This would generally involve documenting bee mortality of bees over time that are foraging in crops sprayed with the active ingredient versus bees in a non treated area. These would be similar to actual bee mortality studies, but actual studies need to be conducted under Good Laboratory Practices which are not possible under most university settings. If preliminary bee mortality studies are performed prior to pre-registration meetings and clear results are available , there would be greater likelihood that the actual studies can be waived. Observational studies (non-GLP) are not likely to be accepted once a study request has been made. In all environmental related data requirements, observational studies may be useful while conducting actual toxicology studies prior to a preregistration meeting may be a waste of resources. The ability to waive bee toxicity studies can also be related to exposure and application timing. Applications of products to soil at planting or other applications that are clearly at times when there is no flowering and bees are not present can also reduce the need for actual studies. If the product is a plant extract, find out if the active ingredient is present in the flower nectar and pollen and if bees forage on the plant or other plants that have the same active ingredient.

DEVELOPMENT OF *ASPERGILLUS FLAVUS* AF36

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Aflatoxins are highly toxic cancer causing fungal metabolites known to cause immune-system suppression, growth retardation, liver disease, and death in both humans and domestic animals. Human exposure to aflatoxins is limited by regulations that prohibit the use of crops containing excess quantities of aflatoxins for foods and feeds. Aflatoxins are regulated in part per billion (ppb) ranges with the maximum allowable level varying with country and intended use of the commodity. The quantity permitted in U.S. foods ranges from 0.5 ppb to 20 ppb. *Aspergillus flavus*, the asexual species responsible for most aflatoxin contamination of many crops, is composed of many genetic groups, called vegetative compatibility groups, that vary widely in several characteristics. *Aspergillus flavus* is not sufficiently aggressive as a pathogen to cause meaningful losses in yield. However, infection of crop components predisposed by stress, insect damage or the environment can result in high aflatoxin levels. Relatively small proportions of a crop infected with highly toxic isolates can result in unacceptable crop aflatoxin content. Isolates of *A. flavus* belonging to different vegetative compatibility groups may produce widely different quantities of aflatoxins and fungal communities resident in different areas frequently vary in average aflatoxin-producing potential. Some naturally occurring isolates of *A. flavus* produce no aflatoxins and are called atoxigenic strains. Certain atoxigenic strains have the ability to competitively exclude aflatoxin-producing strains during crop infection and thereby reduce aflatoxin contamination. One of these, AF36, has been registered as a biological control for the competitive exclusion of aflatoxin producing fungi from cottonseed. The registration process for AF36 that began in 1993 and extended over a decade succeeded in facilitating treatment of over 100,000 acres (Figure 1). The process was greatly facilitated by the IR-4 which served as a liaison and helped prepare and file submissions to EPA.

Many atoxigenic strains are effective at reducing contamination in vitro. However, fewer are effective at reducing aflatoxin contamination during crop infection and in vitro activity does not predict in vivo activity. Laboratory, greenhouse and field plot tests indicated high efficacy of AF36 in competitively excluding aflatoxin producers and reducing aflatoxin contamination of cottonseed and corn (Figure 2). However, it was not until entire commercial cotton fields were treated under an experimental use registration that the full potential of atoxigenic strain technology became apparent. For the registration process the dynamics of population shifts in *A. flavus* communities was monitored carefully. The proportion of *A. flavus* communities composed of the highly toxigenic S strain and of AF36 were monitored prior to treatment, on the crop, and annually after treatment. AF36 was monitored by characterizing numerous *A. flavus* isolates by vegetative compatibility analyses. Treatments caused large reductions in the incidence of aflatoxin producers on treated crops and in soils one year after application and these changes to the *A. flavus* community structures were achieved without increasing the quantity of overall *A. flavus* present (Figure 4). These changes to the structure of *A. flavus* communities influence not only crop aflatoxin content, but the environment as a whole. Propagules (i.e. spores, sclerotia and mycelial fragments) of aflatoxin-producing *Aspergilli* contain large concentrations of aflatoxins. Thus as incidences of atoxigenic strains increase and aflatoxin-producers decrease, and incidences and concentrations of aflatoxins in the soil, air, and throughout the environment also decrease. Thus, use of the pesticide *Aspergillus flavus* AF36 is in the public interest. Long-term and area-wide influences of applications become an emphasis of AF36 development. Many users of atoxigenic

strains seek long-term modifications to the fungal communities in order to reduce risks of aflatoxin contamination in both treated and rotation crops.

The application rate for AF36 is 10 lb/acre. The end use product consists of steam sterilized wheat seed colonized by the atoxigenic *A. flavus* strain AF36. The product is axenic with only the intended atoxigenic strain present. After colonization, the product is dried and stored for up to 9 months prior to delivery to farms. The product is produced in a manufacturing facility operated by a farmer run organization, the Arizona Cotton Research and Protection Council (ACRPC, Figure 3). The process and facility were developed by a partnership between ARS and ACRPC. Quality controls agreed to for the Experimental Registration are maintained as useful insurance of quality product to farmers. For AF36, the registration process was approached as a scientific one. EPA was open to many non-traditional types of information that were ecological in nature. Ecological approaches lead to investigation of the populations of aflatoxin-producing and closely related fungi in natural habitats of the Sonoran desert and in the air above agricultural regions. Several discoveries from these studies have significance well beyond the registration process. *Aspergillus flavus* strain AF36 was found to be endemic not only to agricultural fields, but also to natural habitats. These studies showed that agricultural practices inevitably alter *A. flavus* communities both qualitatively and quantitatively and this can result in average aflatoxin-producing potentials being greater for fungal communities in agricultural fields than for fungal communities in natural habitats. Later, detailed studies showed that soil type, location, and crop rotation all alter the composition and thus the average aflatoxin-producing potential of *A. flavus* communities resident in a given region. Highly toxic *A. flavus* was found in natural habitats along with incidences of deadly aflatoxin levels in samples of native leguminous seeds.

In selecting AF36 as the atoxigenic strain to develop as a commercial biocontrol agent, its efficacy was contrasted with those of other atoxigenic strains in greenhouse and field plot tests. The natural distribution of AF36 on the target crop (cotton) across target regions was also considered. Potential adverse environmental impacts were avoided by not applying *A. flavus* strains to fields in regions where they did not naturally occur. Relative incidence of strains (defined here as VCGs) on the target crop in target regions was taken as a measure of relative adaptation to the crop. It was thought that the most common strains on the crop in these regions would have the greatest adaptation to both the crops and the environments in which the biocontrol needed to work. Atoxigenic strains used for biocontrol need to be highly competitive during crop colonization and infection and throughout the crop environment. AF36 occurred in all the target regions and was the most common atoxigenic strain on the cotton crop throughout these regions. AF36 is an excellent choice for biocontrol strategies based on single atoxigenic strains. However, many other atoxigenic strains also have efficacy. Certain less common strains may be active under specific conditions or in minor components of fields. Natural communities of *A. flavus* endemic to agricultural fields and associated with crops are highly diverse and are composed of many VCGs. After fields are treated with AF36, the resident communities become dominated by AF36. Increased displacement and greater long-term stability of modified communities might be achieved by utilizing mixtures of atoxigenic strains that more closely reflect the complexity of natural *A. flavus* communities. Furthermore, strain mixtures could be customized for various regions, crop rotations, or soil types. The nature of the product may allow the cost of generating such mixtures to be minor. However, the registration process may impede utilizing strain mixtures. Strategies such as atoxigenic strain technology might be particularly limited by pesticide regulations because a continually growing list of strains (reaching perhaps hundreds) may need approval. Instigation of a low cost path to registration of additional strains (similar but not identical to those originally registered) might make further development of atoxigenic strain technology faster and more economically feasible, particularly for the public sector. This would facilitate development and optimization of such technologies for multiple crops, locations, and environments.

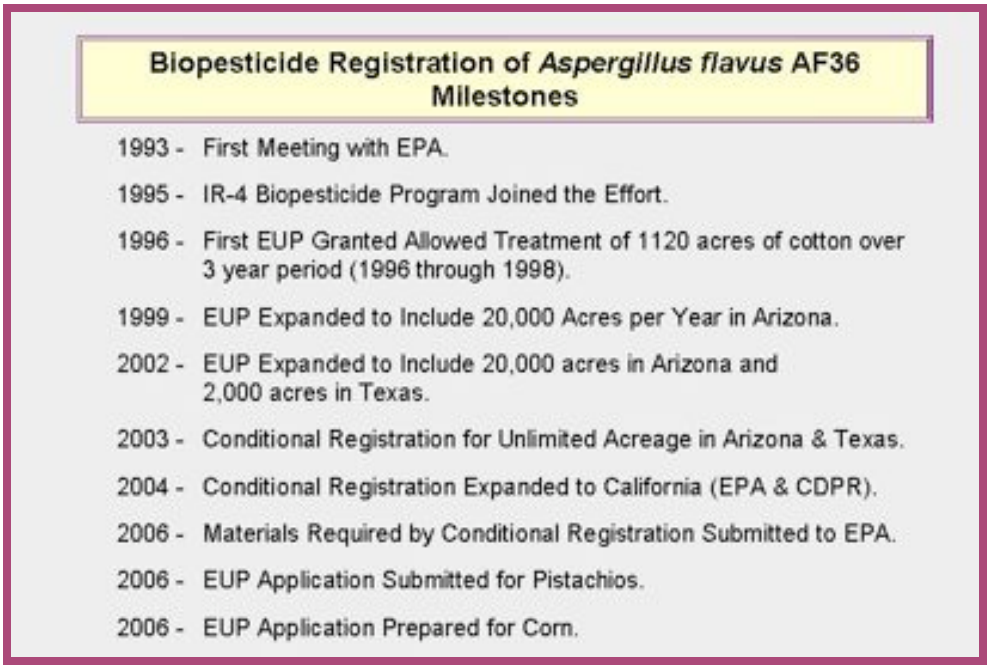

Figure 1. Timeline for the registration of *Aspergillus flavus* AF36. The registration process was undertaken by USDA-ARS with assistance from the IR-4 project. Input from the National Cotton Council and other industry source was important throughout the process. The registrant for the full registration is the Arizona Cotton Research and Protection Council.

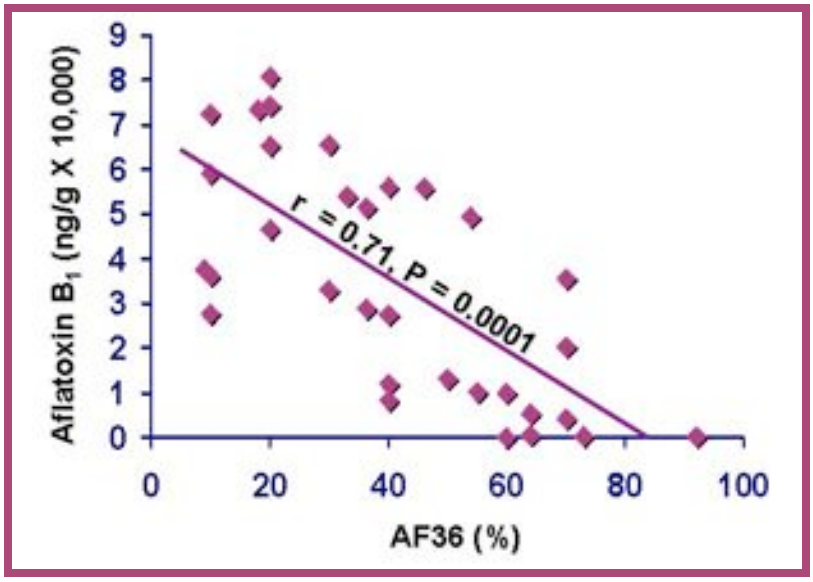

Figure 2. The quantity of aflatoxins in the cottonseed decreases as the percent of the *A. flavus* on the crop that is AF36 increases. Individual points are values from replicate plots either treated with AF36 in various ways or untreated controls. Values are for infected seed. From P.J. Cotty, 1994, Phytopathology 84:1270-1277.

Figure 3. Facility for manufacturing atoxigenic strain material run by the Arizona Cotton Research and Protection Council in Phoenix, Arizona. The process and facility were developed by a partnership between ARS and ACRPC. Photographs: P.J. Cotty.

Composition of *Aspergillus flavus* Communities in Soil of Treated and Nearby Fields in May 1996 Prior to Application of AF36 and in May 1997 One Year After Application

Field type	Fields (#)	AF36 (% *A. flavus*) 1996	AF36 (% *A. flavus*) 1997	S strain (% *A. flavus*) 1996	S strain (% *A. flavus*) 1997	*A. flavus* (CFU/gram) 1996	*A. flavus* (CFU/gram) 1997
Treated	3	4% ab	85% a	52% a	4% d	582 a	365 a
Adjacent	4	2% b	48% b	41% a	18% c	411 a	157 a
Diagonal	4	2% b	16% c	52% a	33% b	61 a	100 a
Other	4	9% a	9% c	43% a	50% a	109 a	98 a

Other	Adjacent	Treated	Adjacent	Other	Other
Other	Diagonal	Adjacent	Diagonal	Other	Other

Figure 4. Proportion of *A. flavus* communities in soil composed of the biocontrol agent atoxigenic strain AF36 prior to treatment (1996) and one year after treatment (1997). Entire commercial cotton fields (approximately 40 acres each) were treated under an experimental use registration. Data on the incidence of the highly toxigenic strain and on the overall quantity of *A. flavus* in the soil is also included. Note that in treated fields, one year after treatment, incidence of the atoxigenic strain is increased and incidence of the high aflatoxin-producing S strain is decreased without increasing the overall quantity of *Aspergillus flavus* in the soil. Unpublished results, P.J. Cotty.

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Elimination of a Primary Filariasis Vector Population at an Endemic Field Site

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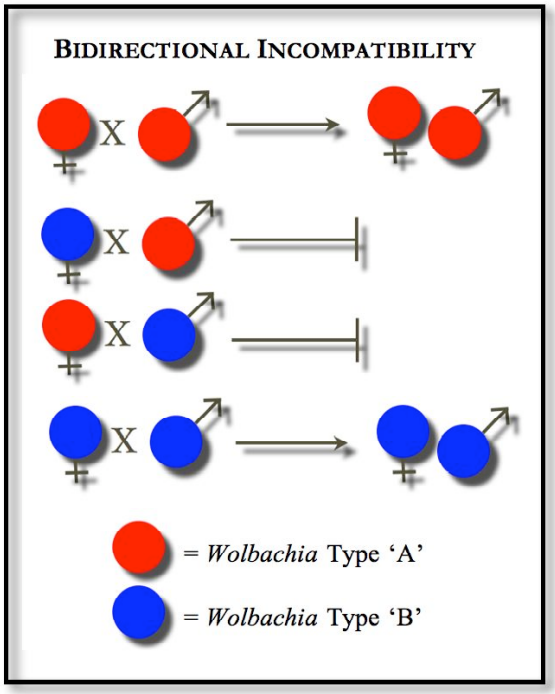
Lymphatic filariasis (Elephantiasis) affects over 120 million people in 80 countries, with 1.2 billion people at risk worldwide. Over 90% of infections are caused by *Wuchereria bancrofti*, for which humans are the exclusive host. The absence of a nonhuman reservoir has prompted an elimination strategy premised upon the hypothesis that transmission can be interrupted by elimination of the microfilariae reservoir via community-wide treatment (Mass Drug Administration, MDA), which is the current focus of the Global Programme for the Elimination of Lymphatic Filariasis. While MDA strategies can be effective, history suggests that elimination of lymphatic filariasis in Polynesia is unachievable without vector control. An example is provided by Maupiti in French Polynesia, where filariasis persists despite decades of constant MDA. The biology of the primary mosquito vector, *Aedes polynesiensis*, has been hypothesized as a primary cause for the lack of MDA success. Since mosquitoes are obligate vectors of *W. bancrofti*, this suggests an additional approach for filariasis elimination: removal of the mosquito vectors will break the disease transmission cycle. Unfortunately *Ae. polynesiensis* currently cannot be controlled, much less eliminated.

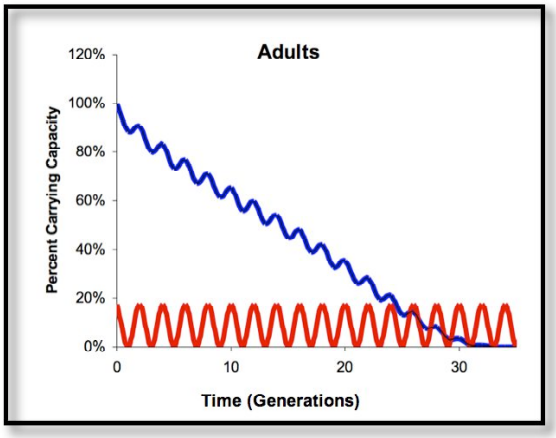
A novel strategy will be described in which releases of male *Ae. polynesiensis* mosquitoes infected with Wolbachia bacteria result in the sterilization of female mosquitoes at a site endemic for filariasis transmission. Wolbachia are maternally inherited, obligate, intracellular bacteria that naturally infect *Ae. polynesiensis*, other mosquito species, and an estimated >20% of insects. Through a mechanism known as 'cytoplasmic incompatibility' (CI), Wolbachia bacteria promote infection spread into mosquito populations by sterilizing female mosquitoes that lack the infection. In natural populations, CI-induced sterility is a transient event, since CI does not occur following Wolbachia invasion and once the population is uniformly infected. In the proposed strategy however, the occurrence of CI-induced sterility will be artificially prolonged by releasing males only. Since Wolbachia is only transmitted through females, the infection does not establish in the field, and the CI-induced sterility persists. By repeating a constant level of male releases, an increasing impact (i.e., Wolbachia-induced sterility) on the *Ae. polynesiensis* population is predicted, resulting in the elimination of the targeted mosquito population. It is emphasized that male mosquitoes do NOT blood feed and therefore are not disease vectors. Furthermore, the proposed strategy employs a naturally occurring bacteria infection and does NOT include genetically modified organisms. The geography of Polynesia will simplify an elimination approach by reducing problems of vector reinfestation via immigration.

Existing data will be presented, describing the generation of the Wolbachia-infected *Ae. polynesiensis* strain and lab assays demonstrating male competitiveness and an ability to sterilize female *Ae. polynesiensis* mosquitoes. A research plan will also be presented,

describing laboratory and field cage tests of the elimination strategy, followed by field trials in which an *Ae. polynesiensis* population is eliminated from an endemic focus of filariasis. Additional planned experiments include the characterization of the targeted field site prior to, during, and following the field trial. Prior to the field trial, experiments will compare the release strain and field population in their fitness, population dynamics and genetic structure, mating competitiveness, and vector competency. An economic model for transitioning from field trials to a vector elimination campaign will also be discussed, emphasizing the economic feasibility of an elimination campaign relative to ongoing vector control in Polynesia. This research is funded through a grant [R01AI067434] from NIH/NIAID.

Relevant to the Workshop, the proposed field trials will provide important risk- and cost-assessment information relevant for future bio-control and transgenic strategies, including: determining appropriate release ratios, estimating the migration and competitiveness of released mosquitoes, monitoring for an unexpected negative ecological impact and assessing the hypothesized beneficial epidemiological impact (i.e., elimination of lymphatic filariasis transmission) resulting from vector population modification. Successful elimination will provide rationale for extending the strategy to a broader geographic range and additional insect species that act as pests and disease vectors.





Predicted elimination of natural mosquito population (blue line, above) resulting from the repeated release of bidirectionally incompatible males (red line, above). Cross pattern of bidirectional cytoplasmic incompatibility is shown in the figure to the left.

APHIS/BRS Regulatory Process for Genetically-Engineered Microbes

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Biotechnology Regulatory Service (BRS), within the Animal Plant Health and Inspection Service (APHIS) of the United State Department of Agriculture regulates, among other genetically engineered organisms, the movement and release of transgenic microbes. The presentation will discuss the regulatory authority for BRS's oversight of transgenic microbes, and how the authority sets the framework for permit data packages. APHIS Form 2000 data requirements and administrative processes for import permits, interstate movement permits and field release permits for transgenic microbes will be presented and discussed.

THE GRAND CHALLENGES IN GLOBAL HEALTH INITIATIVE: GENETIC STRATEGIES TO CONTROL INSECT VECTORS OF DISEASE

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The Grand Challenges in Global Health (GCGH) initiative[1] was launched by Bill Gates in January, 2003, to encourage innovative research that, if successful, could result in scientific or technical breakthroughs that would overcome one or more bottlenecks in an imagined path towards the solution of several significant global health problems. The initiative takes as its model the grand challenges formulated more than 100 years ago by mathematician David Hilbert, a list of important unsolved problems that has encouraged innovation in mathematics research ever since. In October, 2003, the GCGH Scientific Board announced a list of 14 Grand Challenges[2], ranging from creating vaccines that can be delivered more easily to people in the developing world to developing more accurate methods for measuring health status in those regions. Two of the Grand Challenges seek better methods to control insects that transmit agents of diseases particularly relevant to developing countries. One of these deals with development of chemical strategies for making vector populations incompetent to transmit disease agents, or for substantially reducing the prevalence of the vector. The other seeks to develop genetic strategies for a similar purpose.

The GCGH initiative supports three projects aimed at development of genetically engineered mosquitoes to control dengue or malaria, and an additional project aimed at using a life-shortening strain of *Wolbachia* as a form of biological control of dengue vectors[1]. Of the three projects proposing genetic engineering of mosquitoes, one has plans to conduct caged field trials in a disease endemic region within the 5 year award period, i.e. before 2010. These trials, to be conducted under fully caged conditions and using uninfected genetically engineered *Aedes aegypti*, are intended as proof of principle to establish the potential of this strategy with regard to whether genetically engineered mosquitoes can compete with and mate with wild-type mosquitoes in semi-field environments. It is also the intent of this project to develop a pathway for the performance of such studies with regard to: 1) procedures for appropriate conduct of contained field trials, 2) strategies for community engagement and other ethical, social and cultural considerations, and 3) appropriate institutional, national and international approval mechanisms. The first point is currently being addressed by a scientific working group, which is developing a guidance document for contained field trials, including recommendations for physical containment, surveillance and remediation, as well for a phased testing process with points to consider for risk assessment at each phase.

Reference:
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2 Varmus, H., R. Klausner, E. Zerhouni, T. Acharya, A.S. Daar, and P.A. Singer. 2003. Grand Challenges in Global Health. Science **302**: 398-399.

REGULATORY SUMMARY OF AF-36

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Strains of *Aspergillus flavus* produce aflatoxin, a potent microbial compound associated with animal toxicosis and other adverse effects. Aflatoxin contamination of cottonseed causes significant losses for the animal feed industry annually. Few alternatives, if any, exist to control aflatoxin-producing *A. flavus* strains in cotton and other crops. USDA Agricultural Research Service researchers have devised a method to displace toxigenic strains in the agro-ecosystem with more benign strains of the fungus. The AF36 strain of *A. flavus* is one of these benign strains and requires regulatory consideration as a microbial pesticide. The data requirements to support the safety finding for an experimental use permit (EUP) and registration of a microbial pesticide are found at 40 CFR 158.740. There are also guidelines suggesting how the studies should be done (OPPTS Harmonized Guidelines 885 series). The typical course of a registration action includes the review of safety test data supporting an EUP while more specific data, if needed, to justify the commercial registration is developed. The process for AF36 was somewhat different than the typical course for a microbial pesticide registration. Rather than submitting the toxicity/pathogenicity data for a safety determination, a large EUP with extensive monitoring was chosen to address possible increased exposure to this ubiquitous fungus. Given reports in the published literature regarding infectivity of *A. flavus*, there were still concerns about pathogenicity and efficacy to be addressed for the registration decision. The EUP phase was used to generate the fungal ecology information related to AF36 and its levels over the growing season compared to the indigenous *A. flavus* strains. Toxicity/ pathogenicity studies in rodents and other test species were done to address issues related to reports in the literature regarding reports of pathogenicity for this species. The registrant was also required to generate efficacy data since control of microbes producing mycotoxins is considered a claim to control microbes that pose a public health threat. The data submitted and reviewed will be discussed in the context of typical microbial pesticide registration and the flexibility of that process.

DEVELOPMENT OF SYMBIOTIC CONTROL OF PIERCE'S DISEASE

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Pierce's Disease (PD) is a lethal infection of grapevine xylem by the bacterium *Xylella fastidiosa* (*Xf*) for which there is no cure. Control of the glassy-winged sharpshooter (GWSS), the principle vector of PD, is currently the only effective strategy to manage this disease, however it is very costly.

Limiting the spread of *Xf* by rendering GWSS incapable of pathogen transmission or by interfering with the replication of *Xf* in the plant may stop the spread of PD. These goals can be accomplished by genetically modifying bacteria that live in the sharpshooter, the plant, or both in a method called symbiotic control. Symbiotic control seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria.

We set out to apply symbiotic control to Pierce's Disease by carefully considering several criteria that would ensure safety and efficacy of the resulting symbiotic control strain. These criteria were the choice of ecologically-significant yet non-pathogenic symbiotic bacterial species, development of specific anti-*Xylella* effector proteins, an effective means to deliver those proteins, and a method to reduce the likelihood of horizontal transfer of anti-*Xylella* genes from the symbiont to non-target bacteria.

Choice of symbiont. The digestive tracts of field-caught GWSS were screened for the presence of bacteria and several were isolated routinely. One of these was *Alcaligenes xylosoxidans denitrificans (Axd),* a Gram negative pseudomonad-like species that was isolated from GWSS foregut and cibarium. These anatomical regions are precisely the ones that *Xf* occupies in the sharpshooters as it is vectored from plant to plant by the insect. *Axd* can also colonize various plant tissues, including grape and citrus xylem. Importantly, *Axd* is non-pathogenic in insects, plants and healthy humans[1].

Anti-*Xylella* effector proteins and their delivery. Anti-*Xylella* proteins should be as specific as possible for the target organism. Unfortunately, we still know relatively little about the biology of *Xf* so specific targets for anti-*Xylella* factors remain largely unknown. With this in mind, we screened a single chain antibody (scFv) library against intact *Xf* cells to isolate scFvs that bound to the surface of the cell. We were able to isolate one scFv, called S1, that binds to to an unknown target on the surface of Pierce's Disease strains of *Xylella* only. S1 by itself has not shown any anti-*Xylella* activity, but it can be modified to carry anti-bacterial toxins (e.g., peptide antibiotics) that could kill *Xylella* in the plant or its insect vector while still providing the same specificity inherent to single chain antibodies.

Delivery of anti-*Xylella* factors inside the insect vector or grapevines offers another challenge and we have developed two systems to do this. The first is surface display[2]. Surface display is a method to anchor an anti-*Xylella* protein in the outer

membrane of *Axd* where it is exposed on the surface of the bacterium. We used the ice nucleation protein *inaZ* from *Pseudomonas syringae* to anchor the anti-*Xylella* scFv S1 to the surface of *Axd* and could successfully detect it by ELISA and a physical test for ice nucleation ability. Anchored to the surface of the cell, anti-*Xylella* factors have the opportunity to interact with *Xf* should *Axd* come into contact with it in either GWSS or grapevines.

The second delivery system is secretion. Secreted anti-*Xylella* factors have the opportunity to circulate throughout the plant, reaching foci of infection across physical xylem boundaries. Secretion from Gram-negative bacteria, however, is complicated by the fact that these species have two membranes that a protein must cross before appearing outside the cell. One system that seems to have wide applicability is the α-hemolysin autotransporter from *E. coli* [3] This protein is secreted in a single energy-dependent step across both membranes of Gram negative bacteria when the other components of the system are also present (the proteins HlyB, HlyD, and TolC). Fusion of the last 60 amino acids of the protein is sufficient to target any N-terminal passenger protein for secretion. We have successfully secreted functional scFvs using this system in *E. coli* and are currently testing it in *Axd*. Once the system is established it can be quickly adapted to nearly any anti-*Xylella* protein factor.

Suppression of horizontal gene transfer. A critical aspect of any genetically modified organism developed for field release is the suppression of horizontal gene transfer to non-target species. We have taken two complementary approaches to dealing with this challenge. First, we developed a system to incorporate genes into the chromosome of *Axd* based on the transposable element *Himar1*. Horizontal transfer from the chromosome is orders of magnitude less likely than when genes are carried on plasmids. Secondly, we tested a system to actively suppress horizontal gene transfer based on the ColE3 plasmid addiction system. We added the bacterial toxin gene *colE3* (which encodes the antiribosomal protein, colicin) to a plasmid and incorporated the immunity factor for this toxin, *immE3* into the chromosome of *Axd*. When these two factors exist in the same cell, the cell can survive. The presence of the colicin toxin alone kills the cells. We attempted to transfer the colicin plasmid from *Axd* (*immE3*) strains to strains of *E. coli* that either did or did not contain *immE3*. The colicin plasmid was readily transferred to *E. coli* (*immE3*) but we could detect no transfer at all to *E. coli* without *immE3*, indicating that this genetic system can suppress horizontal gene transfer from *Axd* by at least a factor of $1X10^7$ [4]. Tightly linking the *colE3* gene to whatever anti-*Xylella* genes are inserted into the chromosome of *Axd* should eliminate any chance of horizontal transfer to non-target species.

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What Do Risk Assessments of Agricultural Biotechnologies Need to Tell Risk Managers? A Perspective from Economics

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Risk assessments provide a model of how new agricultural biotechnologies generate and propagate risk to human health and the environment. Our preventive approach to regulation (not to mention an aversion to morally reprehensible forms of experimentation) makes uncertainty about those risk inevitable, so that risk assessments need to provide information about uncertainties as well as best estimates of the components of risk generating processes. Risk assessors draw on the expertise of individual scientific disciplines to obtain the best prospective information about each component of the processes that generate risk. A frequently neglected problem is how that information should be combined into an overall estimate of risk. Overlooking that question is unfortunate, because how the final estimate of risk will be used has profound implications for how the components of the risk generating process should be estimated.

How the components of models of risk generating processes should be estimated and assembled is best understood from the perspective of the model user, that is, a regulatory agency charged with making decisions about whether new biotechnology products should be introduced and, if so, what (if any) restrictions should be placed on their use. The model introduced into the literature by Lichtenberg and Zilberman and elaborated further by Lichtenberg, Zilberman, and Bogen, Ziven and Zilberman, and Lichtenberg and Penn provides a useful conceptual framework for thinking about how standard types of risk assessments and a standard approach to accommodating uncertainty about risk can be used in cost efficient risk management. In its simplest form, this model divides components of the risk generating process into three categories—those governing the introduction of a potentially hazardous material into the environment, those governing the environmental fate and transport of that material, and those influencing susceptibility of humans or other organisms to adverse effects from that material. Regulatory actions and responses of economic agents can affect all three types of components. Regulatory agencies are assumed to minimize the cost of achieving an acceptable level of risk with a reasonable margin of safety, which corresponds to a form of risk management based in classic statistics in which regulatory actions are aimed at the upper limit of a one-tailed confidence interval.

This conceptual approach has some important implications regarding risk assessment and regulation for new agricultural biotechnology products.

First, a consistent approach to decision making requires consistent treatment of adjustments for uncertainty and is possible only if assessments of each component of risk are transparent, conducted using consistent methodologies, and provide best estimates

and estimates of uncertainty separately. The upper limit of a one-tailed confidence interval can be expressed in terms of a weighted sum of the estimated means of the components of the risk generating process and the standard deviation of the overall estimated risk (which depends on the variances and covariances of those components), with weights determined by the desired confidence level, that is, the desired margin of safety. Instead of being adjusted for uncertainty in a systematic fashion, though, risk estimates are frequently constructed by applying arbitrary safety factors or other ad hoc adjustments for uncertainty to each component. As has been widely discussed in the literature, the resulting risk estimates have no consistent statistical meaning, distorting comparisons of relative risk and making it impossible to compare the effects of regulation across new biotechnology products.

Second, cost efficient regulation consists of a portfolio of actions, some specializing in reducing risk on average and some in reducing uncertainty about risk. Limits on introduction or usage are aimed largely at reducing risk on average; information gathering, including pre-regulatory testing and post-introduction monitoring, are aimed largely at reducing uncertainty about risk. It is often the case that uncertainty is extensive and/or concerns about uncertainty are high even though best estimates indicate low risk. In such cases it will be cost efficient to emphasize policies that reduce uncertainty about risk, including larger-scale field testing and extensive post-introduction monitoring, while de-emphasizing policies that reduce risk on average, such as severe restrictions on introductions of new technologies.

Third, the model provides clear conceptual foundation for incorporating findings of social science research regarding risk perceptions and reactions of producers and consumers to regulation into regulatory decision making. Standard quantitative risk assessments assume that all components of risk generating processes follow physical or biological laws. They typically ignore human behavior, including behavioral responses to new products and to regulation of those products. But firms' compliance with regulations is known to be imperfect because firms balance expected penalties for non-compliance against the costs of actions taken to meet regulatory requirements. Stricter safety regulations can change consumer behavior, for instance, by inducing consumers to take fewer personal precautions. Models positing perfect compliance will thus tend to underestimate risk, suggesting that social science research should be given a greater role in risk assessments.

Fourth, expanding the model to accommodate multiple heterogeneous sites indicates that one size fits all regulation tends to be inappropriate when heterogeneity is significant. One type of situation of special interest is when it is possible to distinguish cases where there is no need for regulation because risk always falls below the acceptable level with the desired margin of safety. The potential for such cases speaks to the need to tailor regulation to the circumstances of different geographic areas rather than imposing uniform restrictions.

3B.4 ABSTRACT

GENETIC MODIFICATION OF INSECTS OF MEDICAL IMPORTANCE: PAST, PRESENT AND FUTURE

MARCELO LORENA, JOHNS HOPKINS

Together with AIDS and tuberculosis, malaria is among the deadliest infectious diseases in the world and responsible for an estimated 2 million deaths, mostly of African children, every year. Also worrisome, is that the number of cases has been increasing, not decreasing. This suggests that the weapons available to fight malaria is not sufficient and underscores the need to develop new approaches to complement the existing ones.

Unlike AIDS and tuberculosis, transmission of *Plasmodium*, the causative agent of malaria, strictly depends on an intermediate host, the mosquito vector. Consequently, the mosquito stages of parasite development are potential week points that can be targeted for disease control. Several laboratories, including ours, are exploring the possibility of using genetic modification of the mosquito to render it incapable to sustain parasite development. The presentation will review past efforts to develop transgenic mosquito technology, the present status of the field and future prospects.

Insertion into the mosquito germ line of genes that render the mosquito refractory to the parasite has three essential requirements: the ability to introduce genes into its germ line, the characterization of promoters that can drive foreign genes in the appropriate mosquito tissue and at the appropriate time, and the identification of "effector genes" whose products interfere with parasite development without affecting mosquito fitness.

Germ line transformation. The first multicellular organism ever to be genetically transformed is an insect, *Drosophila melanogaster*. Although this was reported in 1982, genetic transformation of *Ae. aegypti* was reported only 16 years later (1998) and that of an anopheline mosquito (*An. stephensi*) in 2000. These advances made feasible the introduction of foreign genes into the malaria vector.

Promoters. Development of *Plasmodium*, the causative agent of malaria, occurs in three mosquito compartments: midgut, hemocoel and salivary glands. Characterization of promoters (and associated regulatory sequences) that drive expression of proteins in the first two compartments occurred in 2000 while a promoter for salivary gland expression was just reported.

Effector genes. There have been several effector genes characterized, including the SM1 peptide that blocks parasite invasion of the midgut and salivary gland, phospholipase A2 that interferes with midgut invasion by unknown mechanisms, and monoclonal antibodies that recognize parasite surface proteins.

In conclusion, experiments conducted in the past decade demonstrate that it is feasible to genetically modify mosquitoes to render them refractory to the malaria parasite. The next

big challenge is to devise means to introduce the transgenes into mosquito populations in the field. One factor that will influence the success of such endeavor is the fitness of the genetically modified mosquitoes. In contrast to reports from other laboratories, we found that a transgene by itself does not impose a fitness load to the mosquito. Moreover, we found that when mosquitoes are fed with *Plasmodium*-infected blood, transgenic mosquitoes may actually have a fitness advantage over their non-transgenic counterparts.

Several approaches have been suggested for driving genes into populations, including the use of transposable elements, Wolbachia and meiotic drive. Implementation of either of these will require major technical hurdles to be overcome and these are unlikely to be solved in the near future. For the short term, we feel that an alternate approach – paratransgenesis - offers considerable hope. The mosquito, as all higher organisms, carries bacteria in its gut and the number of these bacteria increases dramatically after a blood meal (nutrients stimulate bacteria multiplication). Moreover, the bacteria are in the same compartment - the midgut lumen - where the most vulnerable stages of *Plasmodium* development take place. Instead of genetically modifying the mosquito, we propose to modify the mosquito's midgut bacteria to produce the anti-*Plasmodium* proteins (paratransgenesis). Initial experiments indicate that this approach may be feasible. The advantages of such paratransgenesis approach include its low tech nature which is important for use in disease endemic countries, ease of expression of any combination of effector proteins, ease of changing effector genes if resistance or other problems develop, and the ability to concomitantly target multiple mosquito species with the same recombinant bacteria. One major technical problem that needs to be solved is to devise a method to introduce the recombinant bacteria into wild mosquito populations. Possible solutions for this potential obstacle will be discussed.

CHESTNUT BLIGHT BIOLOGICAL CONTROL: A TRANSGENIC MICROBIAL APPROACH

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The early 20th century chestnut blight epidemic in North America demonstrated how destructive an invasive species can be to a susceptible host population. The result was the loss of what is estimated to be several billion American chestnut trees. Scientists of the early1900s determined the causal agent was a fungus, *Cryphonectria parasitica*, and declared there was no way to stop its invasion. This organism, a bark pathogen of Asian origin, initiates infections at wound sites. The invading fungus grows through healthy bark tissue eventually girdling the stem so that all tissues beyond the infection die. Fortunately, the American chestnut has survived by producing sprouts from root systems that have remained alive at sites that once supported remarkable stands of American chestnut. The pathogen also has survived on this population of sprouts.

In the early1930s, the same disease was discovered on European chestnuts growing in Italy. Unlike the North American epidemic, some trees survived the disease and rather than being cankered by lethal strains of the fungus the infections were swollen, more superficial and not lethal. When grown in culture strains that produced these infections had reduced pigmentation and sporulation, and resulted in small, non-lethal infections when introduced to healthy chestnut bark. A cytoplasmically-borne, double-stranded (ds)RNA that is transmissible via hyphal anastomosis has since been identified as the agent responsible for these altered traits. Strains so infected have been termed hypovirulent and the membrane-bounded dsRNAs that infect them, hypoviruses. Several dsRNA's since have been identified and characterized including ones that are associated with the recovery of American chestnut in Michigan a situation similar to what has occurred in Europe.

The phenomenon of transmissible hypovirulence has been an appealing option for disease management since its discovery. Molecular technologies now have permitted the cloning and sequence determination of several dsRNA's that have been associated with hypovirulent strains. This in turn has allowed the development of full-length infectious cDNA clones. Remarkably, transformation of virulent strains by the infectious clones produces the complete hypovirulent phenotype and also launches a cytoplasmically replicating dsRNA. Not only do these transfected strains act like the naturally occurring hypovirulent strains but they also are capable of transmitting the genetic elements responsible for hypovirulence via the sexual reproductive cycle. Because significant levels of biological control have not occurred either naturally or when hypovirulent strains have been introduced, the approach of utilizing transgenic strains appears to offer significant benefits for biological control. With naturally occurring hypovirulent strains, the formation of viable anastomoses that permit the transmission of dsRNA's is limited to strains that are closely related in their vegetative compatibility. One benefit of transgenic

strains is their ability to transmit the integrated cDNA into sexual spores; this distributes the cDNA to a variety of vegetative compatibility types. There are additional benefits relative to inoculum production. Nearly all asexual spores of transgenic strains are hypovirus carriers In contrast to some naturally occurring hypovirulent strains that produce low populations of hypovirus-laden asexual spores. Similarly, when crosses between transgenic and wild-type strains occur, one half of the sexual spores carry hypovirus, a feature that does not exist when cytoplasmically-borne and wild-type strains mate.

Most research on microbial biopesticides relative to fungi focuses on the concepts of competition or antagonism where one organism is used to control another. The potential usefulness of technologies involving transgenes, that alter the virulence of economically damaging microorganisms, largely remains an untested yet potentially a useful technology. Certainly the risk of using such technologies needs to be assessed for each microorganism and its infectious agent. However, transgenes are an integral part of their host's genetics and thus the risk they pose of escape into the environment requires consideration. Clearly, any organism that benefits competitively from genetic alteration is of concern and those risks need to be quantified as best possible. With the chestnut blight/hypovirulence system, the benefits derived by enhancing the spread of detrimental hypoviruses among an exotic fungal pathogen would appear to outweigh any measurable risk.

IMPACT OF REGULATORY ACTIVITY ON EMERGING COMPANIES

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According to bcc Research, biopesticides comprise about 2.5% of the global pesticide market (approximately $700 million) and are expected to reach $1 billion by 2010. According to CPL Business Associates, the 2005 market for microbial biopesticides was $268 million of which $109 million are sales in North America. CPL projects a rise from $268 million to $750 million by 2015. While good figures on the growth of biopesticides are hard to find, there is no doubt that the biopesticide market is growing faster than the chemical pesticide market, which is declining. The increase in biopesticides is driven by the removal of some chemical pesticides from the market, global food production that requires residue management, worker safety and labor management – the ability of growers to save costs with short worker re-entry periods, resistance management and the increase in organic farming and IPM. The biopesticide industry (see wwwbiopesticideindustryalliance.org) is attractive for entrepreneurs and investors because biopesticides can provide good growth prospects and are less capital-intensive than chemical pesticides and genetically engineered crops. However, given the significant market risks that biopesticide companies face in agricultural markets, such as commodity price swings, weather, overcoming customer misperceptions, and slow product adoption (relative to other industries), any regulatory hurdles, including inconsistency and unpredictability of the product approval timeframe only inhibit new start ups and investment in biopesticides. Regulatory delays and hurdles can cripple a fledging biopesticide company and significantly impact company growth and even survival. The passage of the Pesticide Registration Improvement Act (PRIA) in 2004 by Congress, which requires registration approval within certain specified timeframes in return for user fees, increased the predictability of approval timelines for biopesticides (and chemicals). This has increased innovation and helps the biopesticide industry bring more products to market. It also increases product improvement (i.e, better formulations) and label expansion because of shorter timelines than they were before PRIA. The speaker will provide an entrepreneur's experiences in discovery, development and marketing of biopesticides and examples of the impact of regulations on biopesticide companies she founded. While regulatory is still critical to a fledging biopesticide company, policies, grants and programs affecting market adoption will have greater impact on the growth and success of biopesticides.

A Risk Assessment Framework for Paratransgenesis

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Paratransgenesis is a novel approach to control of vector-borne disease. In the paratransgenic approach, symbiotic bacteria of a disease-transmitting arthropod vector are genetically transformed to export molecules with activity against a targeted pathogen. We developed a system for control of Chagas disease using the vector *Rhodnius prolixus* and its symbiont, *Rhodococcus rhodnii*. In this system *R. rhodnii* is genetically transformed to express cecropin A, an insect immune peptide with activity against *Trypanosoma cruzi*, the parasite causing Chagas disease. Field application of the paratransgenic approach would involve environmental release of a genetically modified bacterium, an event associated with potentially significant risks. We have developed a comprehensive framework for evaluating risks associated with paratransgenic disease control. We contend that the principles informing this framework can be applied to a spectrum of transgenic disease interventions.

For purposes of explanation, our risk assessment framework will be presented in four stages. The first stage involves identification of adverse outcomes. Here adverse outcomes were identified and considered for assessment of risk on the basis of four criteria: (1) documentation in the scientific literature as a hazard associated with introduction of a novel or genetically modified organism, (2) concern expressed by regulatory, advocacy, or scientific organizations, (3) biological plausibility, and (4) magnitude of effect.

This process selected four adverse outcomes for assessment: toxicity, fitness alteration, transfer to non-target organisms, and transgene instability. In the second stage of the framework, these outcomes were assessed for risk. Assessments were designed and executed pursuant to the following objectives: (1) evaluation under conditions of maximal probability, (2) laboratory simulation of environmental conditions, (3) scale invariance, and (4) uniform spatial and temporal extension. Examples of these will be discussed.

Initial assessment of the risk of adverse outcomes perforce treats these as discrete events detectable in identified populations. There are, however, potential impacts of the paratransgenic approach not amenable to this methodology. Release of large numbers of engineered microbes has the potential to disrupt ecosystems by displacing established organisms, decrementing biodiversity, and altering equilibria of microbial communities. Assessment of these impacts involves biotic communities and requires investigation in

the context of microbial and vector ecology. Therefore, assessment of community ecology impacts is the focus of the fourth stage of our risk assessment framework. The emphasis here is on the extrapolation of assessment of discrete events to the community level using cognate methodologies. Examples of environmental simulations, predictive models, and post-release monitoring and rescue strategies will be presented.

The fourth and final stage of our risk assessment framework involves stakeholder oversight. Fellow researchers, regulatory personnel, public health officials, and members of at-risk groups all have a stake in risk assessment of paratransgenic intervention. Presenting results of a risk assessment program in a setting where dialogue can take place will facilitate incorporation of the concerns and insights of these groups in an ongoing risk assessment program. A proposal for a forum for constructive dialogue between researchers, regulatory agencies, advocacy groups, and representatives of at-risk groups will be presented. Principles of milestone-gated communication will be reviewed and illustrated with examples. How such a forum could be used to facilitate transfer of the research and assessment apparatus to stakeholders in the endemic region will be discussed.

The concluding section of the presentation will emphasize the fundamentally heuristic nature of transgenic risk assessment. Special attention will be paid to the inverse relationship of efficacy and risk. The importance of establishing a minimum effective release (MER) will be illustrated. The potential for applications of network theory models, remote sensing data, and nanotechnology in risk mitigation will be introduced.

RISK ASSESSMENT RESEARCH FOR THE PD PROJECT

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Pierce's disease (PD) is described by Dr. Lampe in an earlier session. It is a disease of grapevines caused by a specific strain of the bacterium, *Xylella fastidiosa*, a xylem-limited bacterium that is acquired by xylem-feeding leafhoppers called sharpshooters carried in the pre-cibarium of the mouthparts/foregut and is transmitted readily as the sharpshooters move and feed. The PD strain of *X. fastidiosa* has no effect on any other plant so far as we know. PD is endemic in the southeastern USA and northeastern Mexico where the pathogen appears to have co-evolved with the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS). Although the pathogen has been present in California for many years, native sharpshooters are not capable of supporting epidemics. This changed with the arrival of GWSS. We do not know when it arrived, but I noticed large numbers of GWSS feeding on yucca in my yard in Riverside in 1988.

The first hint of trouble from GWSS occurred when the insect began transmitting a strain of *X. fastidiosa* to oleanders causing an epidemic of oleander leaf scorch in southern California. This was followed by an outbreak of PD in vineyards in the winegrowing area of Temecula at the end of the 1990s. Soon growers and their bankers in Temecula generated emergency industry, federal and state funding for control and a cure.

If there was a cure for PD, there could be a thriving winegrowing industry in Florida and Texas. In fact, Texas has a struggling wine industry north of San Antonio on the fringe of the pathogen and GWSS distributions and Florida has a very modest effort.

The PD in California is now controlled by an expensive chemical treatment program and an equally expensive and inconvenient quarantine program aimed at the large local nursery industry with active inspection and chemical treatment protocols for nursery stock that is shipped from southern California. Both measures target GWSS, which has so far been contained south of Fresno. We have learned that citrus near vineyards is the highest risk condition for infesting vineyards and spreading the pathogen causing PD in the spring; therefore, the control program treats citrus to protect vineyards. The citrus and grape industries have to get along to make this work and the treatments have to be compatible with the outstanding biological control programs in place in California, but one can't see this going on for a protracted period of time. Eradication of GWSS is not considered feasible in the foreseeable future. On top of this, we do not know exactly what causes PD in the grapevine, nor the exact method of transmission other than physical. Knowing the entire genome of *Xylella* hasn't provided a control method.

Since a solution is demanded, we proposed symbiotic control. This requires new technology never tired before, which is why we require interaction with regulatory officials. Symbiotic control targets the act of transmission of the pathogen by the sharpshooter in a form of competitive displacement. We identified a preliminary symbiont to test, *Alcaligenes xylosoxidans denitrificans*, (*Axd*) which has some of the necessary properties. It was isolated from the pre-cibarium of GWSS, where it attaches alongside and has access to the pathogen. It is a xylem-limited bacterium, again has access to the pathogen. It is smaller that the pathogen, fully 1/10 the physical size of PD-*Xylella*. This attracted enough USDA-APHIS funding to start a 4-member team.

Dave Lampe provided the first genetically transformed *Axd* with *egfp* and then *Ds*Red marker genes. Dave also found an antibody that seemed to recognize the coat protein of the pathogen, S1. We tested a phage-antibody version of this antibody and found that it disrupted the transmission of the pathogen by GWSS. At this time I applied for a permit to do field trials with support from the California grape and wine industry; past experience suggested the sooner the better. It took a while to learn that EPA was the regulatory body; it took EPA a while to figure out which work group was responsible, but they eventually read that *Axd* was found in nosocomial infections in lungs of patients. After a meeting of the EPA SAPs, severe restrictions

were placed on field tests (we had to burn the grapevines at the end of the test). This restriction was not compatible with the BL-1 level of laboratory handling approved by the UCR BioSafety committee. It was also non-negotiable (there was no mechanism to explain the ruling nor to appeal). Bill Schneider warned that *Axd* could well suffer the same fate as *Burkholderia cepacia*, which was dropped from developed due to similar SAP concerns.

This ruling affected what research we did from then on. I asked graduate student Jennifer Parker to do comparative nucleotide identification (a logical step anyway). That cost about 2 man-years. One third of our entire research effort went into identifying the fate of R*Axd* in grapevine, soil and the entire team was distracted by questions of making *Axd* safe rather than making it work to prevent PD. Dave Lampe was distracted from making an *Axd* version that had both the marker gene and his S1 antibody (so-called S1-R*Axd*). Ravi Durvasula then asked Lampe and I to join him in a CSREES-BRAG grant proposal to address risk assessment issues. We obtained a high rating and then the agency asked us to cut the request in half so they could fund 20% of the applications in that round. This allowed an abbreviated look at horizontal gene movement and design of *Axd* versions that would self-destruct (and presumably be more acceptable to EPA) and took us further still away from PD.

Our consultant, Frank Richards, inventor of the symbiotic control strategy for Chagas disease, then devoted considerable time trying to get workers in the cystic fibrosis field to consider the use of *Axd* as a delivery vehicle to clear lung infections of pathogens. His calls to the CF Foundation were not returned and he had to abandon the effort. This proposed new use of symbiotic control still hangs in the air.

In the end we learned that R*Axd* disappeared quickly from commercial grapevines and would not colonize soil unless the soil was first sterilized (in other words our R*Axd* was not competitive). We suspected that our *Axd* was not the same as the one found in fluids in hospitals because of the extreme differences in niches occupied. We will almost certainly pick another symbiont to deliver the anti-PD strategy for ecological reasons, which means the entire interaction with EPA was necessary only for the experience. One thing was clear, no hazard from *Axd* was every identified, which resonates with UCR BioSafety BL-1 rating. And EPA has a better idea how to deal with symbiotic control, at least this application.

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History of Paratransgenesis and a note on its Bioregulation.

Paratransgenic methods are used to modify infectious pathogen-mediated diseases in both plant and animal hosts. Populations of symbiotic bacteria present in the host are genetically modified to express and export in the host, antibody fragments or peptides that have a deleterious effect on the pathogen population. The conditions necessary for successful exploitation of these methods are considered and it is stressed that these methods are adaptations of naturally occurring processes, which have undergone naturally occurring evolutionary processes. Bioregulation of these processes will be most effective if it also follows, gradual evolutionary principles rather than an initial demand for absolute safety, which is usually an elusive goal.

Brief Abstract by Frank.F.Richards.

Regulatory Procedures for Transgenic Insects

Robyn I. Rose, USDA, Animal Plant Health Inspection Service, Biotechnology Regulatory Services

Research related to transgenic arthropods and insects is mostly in early stages of development. There is research underway with a variety of organisms to develop appropriate transformation methods and have focused on transposable elements such as *piggyBac* and paratransgenesis. Selectable markers such as enhanced green and red fluorescent proteins (EGFP and DsRed) are being used to evaluate transformation efficiency. Arthropods have been genetically engineered to be resistant to pests and disease (e.g., honey bees), produce pharmaceuticals (e.g., silkworms), prevent transmission of plant diseases (e.g., glassy-winged sharpshooters, planthoppers), prevent the spread of human and animal diseases (e.g., mosquitoes, kissing bugs, sand flies, tse tse flies, house flies, blowflies, stable flies), with autocidal traits (e.g., pink bollworms and medflies), and as biological control organisms (e.g., mites).

There are several laws that provide regulatory authority for transgenic insects most of which fall under USDA/APHIS. The Plant Protection Act (PPA) (2000) provides regulatory authority to detect, control, eradicate, suppress, prevent, or retard the spread of plant pests. Exotic organisms such as insects imported for use as biological control organisms are regulated under the PPA. Transgenic arthropods that are plant pests (e.g., pink bollworm or medfly) or indirectly affect plant pests (e.g., biological control organisms) are regulated under the PPA. The Animal Health Protection Act focuses on the prevention, detection, control, and eradication of diseases and pests of animals. Genetically engineered (GE) arthropod pests of humans and animals such as mosquitoes that are made incapable of vectoring disease are regulated under the Animal Health Protection Act. The Virus-Serum-Toxin Act (VSTA) is intended to assure the safe and effective supply of animal vaccines and other biological products. Arthropods such as mosquitoes that have been genetically engineered to vector disease vaccines would be regulated under the VSTA as well as the AHPA. The Honey Bee Act gives APHIS authority to regulate importation and propagation of honey bees. Additional authority for transgenic insects falls to the Food and Drug Administration (FDA) which regulates new animal drugs under the Federal Food Drug and Cosmetic Act (FFDCA). The Environmental Protection Agency may regulate environmental releases of arthropods with a symbiotic relationship with a genetically engineered microbe under the Toxic Substances Control Act (TSCA) or the Federal Insecticide Fungicide and Rodenticide Act (FIFRA).

An APHIS permit is required for the importation, movement or environmental release of transgenic insects. Appropriate containment or confinement of the transformed organism is required whether the organism is released, imported or moved interstate. A permit for environmental release will require a risk assessment, a formal NEPA analysis (in some cases by preparation of an Environmental Assessment (EA)) that is shared with the public, publication of the availability of the EA in the Federal Register, a public comment period, final deliberations, and then if appropriate, an issuance of a Finding of No Significant Impact (FONSI) and approval of the permit

(http://www.aphis.usda.gov/brs/arthropod_discuss.html). Permits have been issued by BRS for caged and open-field releases of transgenic pink bollworms and predatory mites (http://www.aphis.usda.gov/brs/arthropod_release.html).

When an application is received, it is evaluated for completeness for the purpose of doing a risk assessment. If it is found deficient, the applicant is informed what information is needed and time is allowed to provide the information. The main purpose of the risk assessment is to determine if genetic alteration changes ecological or environmental properties of the organism. Such potential risks associated with the release of a transgenic arthropod or other invertebrate could include displacement of native populations, change in host or prey utilization, change in distribution, effects on endangered or threatened species, transfer of DNA to other organisms, or, if one of the characteristics of the transgenic arthropod was increased resistance to herbicides or pesticides (http://www.aphis.usda.gov/brs/arthropod_discuss.html).

A confined field trial is where the candidate arthropod is prevented from becoming established and spreading. Confinement may be by physical barriers such as screen cages, pesticides, cultural control, and biological measures such as induced sterility or pheromone traps. Confined field tests can provide important information before unconfined release is requested and may be useful to observe changes in biology, ecology, and behavior of the transgenic form compared to the parental form (http://www.aphis.usda.gov/brs/arthropod_discuss.html).

EPA Regulatory Process for Microbial Pesticides

William R. Schneider, US Environmental Protection Agency

The first microbial pesticide was registered in 1948. As we subsequently evaluated more microbial pesticides we realized we needed different kinds of tests than the standard toxicity and environmental fate tests required for conventional chemical pesticides. We began development of unique data requirements and tests in the 1970s. We consulted scientific experts and published regulations for how to register microbial pesticides and guidelines on how to perform the testing.

The data requirements that are currently in effect were published in 1984. We are just now finishing a revision to these data requirements that better reflects the regulatory process that we have evolved over the years since then.

The revised definition for a microbial pesticide now reads:
A Microbial pesticide is a microorganism intended for preventing, destroying, repelling, or mitigating any pest, or intended for use as a plant regulator, defoliant, or dessicant, that:

(1) Is a eucaryotic microorganism including, but not limited to, protozoa, algae, and fungi;
(2) Is a procaryotic microorganism, including, but not limited to, bacteria, and archaea;

or

(3) Is an parasitic replicating microscopic element, including but not limited to, viruses.

There are now over 78 registered microbial pesticides. The BioPesticides and Pollution Prevention Division was established in 1995 to handle the increasing numbers of these specialized pesticides.

Pesticide regulation is handled under several laws, the Federal Insecticide, Fungicide, & Rodenticide Act (FIFRA), and the Federal Food, Drug, & Cosmetic Act (FFDCA), which were both modified by the Food Quality Protection Act, and the Pesticide Regulatory Improvement Act, which sets fees for regulatory actions. The laws authorize publication of rules on how the laws will be administered. These rules are published as regulations, which are first announced in the Federal Register and later published in the Code of Federal Regulations, “Title 40” Pesticides. Regulations of particular relevance to the regulated community are the following:

40 CFR 152.3 Definitions
40 CFR 152.15 Defines pesticides that need registering
40 CFR 152.20 Exemptions for biocontrol organisms
40 CFR 152.158 Data requirements
40 CFR 152.172 Experimental Use Permits
40 CFR 152.180 Tolerances (residues on foods)

In addition to regulations, we have published Guidelines, which are recommendations on how to perform the studies listed in the data requirement regulations and can be found on our website at www.epa.gov/opptsfrs/home/guidelin.htm .

We use the standard pesticide risk assessment paradigm for microbial pesticides, in that we use hazard information and exposure information to assess risk. The hazard information for microbials includes both toxicity and pathogenicity. We need to assess risk for non-target organisms, humans, domestic animals, and have to consider potential effects on endangered species.

The microbial pesticide data requirements are published in 40 CFR 158, which is currently being updated. The proposed revised data requirements were published in the March 8, 2006 Federal Register. Additional regulatory assistance information can be found at our website, www.epa.gov/pesticides/biopesticides .

Burkholderia cepacia ; TSCA Regulatory aspects

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In 2001, the Cystic Fibrosis Foundation approached EPA with a petition under a provision of the Toxic Substances Control Act to regulate members of the "Burkholderia cepacia complex" (BCC) used for commercial applications (not including pesticides). EPA responded by evaluating the risks of such organisms in the context of uses subject to TSCA rules. The assessment considered a range of issues associated with this diverse group of widely distributed bacteria. Members of this group have some potential utility for such things as bioremediation and inclusion in drain cleaner mixes, based on the extensive set of biocatalytic enzymes they frequently possess. However, many isolates of this group, especially those used to define new species within the complex, are found to be associated with serious infections of susceptible human populations including but not limited to CF patients. This talk will address the characteristics of the BCC which simultaneously contribute to its potential as a remediation agent and as an opportunistic pathogen. The bases for a determination by EPA to limit use of these agents for TSCA purposes will be described, as well as more recent scientific observations about the characteristics of the group.

DEVELOPMENT OF TRANSGENIC PINK BOLLWORM FOR USE IN AN AREA-WIDE GENETIC CONTROL PROGRAM: PERSPECTIVES FROM THE LABORATORY ON TECHNICAL AND REGULATORY ISSUES.

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The best known genetic control technology, the sterile insect release technique (SIT), uses radiation to cause genetic damage or sterility to the gametes of the release insect so that all of the progeny from a mating with the wild target pest are nonviable. Use of SIT against pink bollworm (PBW) has proven to be very successful and has been used for over 30 years to protect a large cotton growing area in the Central Valley of California. However, PBW require a high dose of radiation to cause sterility, which causes a significant decline in the performance of the insect. This effect of radiation makes high release rates necessary. As the demands of an expanding PBW eradication program in the western cotton belt put more pressure on limited resources, a more competitive release insect is needed.

To improve the effectiveness of PBW genetic control, two control methods with transgenic insects are in development: a low dose F-1 sterile release strategy using genetically marked PBW (e.g., GFP from jellyfish, DsRed from coral); and the release of a conditionally lethal PBW strain as an alternative or supplement to sterilization by irradiation. Because these two control strategies use either a low dose or no irradiation, the release insect is more competitive allowing lower release rates to be used.

Results from laboratory, field cage, and open field testing of several transgenic strains will be presented along with the regulatory history of the project. Future progress towards potential use in the eradication program will depend on meeting both technical and regulatory challenges.

GE and non-GE fungi: Risk Assessment.

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A major deterrent to the development of fungi as pesticides has been that it can take 5 to 15 days post-infection to kill the targeted pest. This not only makes them poorly competitive, but also limits industrial investment in application and formulation technologies for advanced efficacy. Consequently any consideration of the suitability of a pathogen for commercial development inevitably leads to the possibility of improving its performance. Ultimately, various traits of flingal pathogens, including host range, production capacity, stability and virulence, might be enhanced through genetic manipulations. Unfortunately, host specific strains best adapted to an IPM program in particular kill slowly and produce fewer toxins than generalist strains that could potentially kill natural predators as well as target pests. Presumably strains that are not specifically adapted to subvert/avoid/overcome the immune response of a particular insect are best served by achieving a rapid kill with toxins. An adapted strain may optimize utilization of host nutrients and production of infectious propagules by growing within the living host. Adding new genes to the fungus that will allow it to kill the insect host more quickly is a solution. Attractive initial candidates for this approach include cuticle-degrading enzymes and toxins that are encoded by single genes as they are highly amenable to manipulation by gene transfer.

Recombinant Metarhizium strains that constitutively overexpress the subtiisin protease Pria have improved pathogenic qualities at all stages of infection. In contrast to the wild-type, transgenic strains continued to produce Pri in the haemocoel of Manduca sexta caterpillars following penetration of the cuticle. This caused extensive melanization in the body cavity, and early cessation of feeding. Insects killed by transgenic strains and extensively melanized were very poor substrates for fungal growth and sporulation. This reduces transmission of the recombinant fungi, which assisted in obtaining permission for a field trial. The trial occurred on a patch of cabbage with an engineered hypervirulent strain carrying extra protease genes plus the gene for EGFP1 (a variant of the green fluorescent protein). The gfp gene is driven by a constitutive promoter and the cytoplasmically located protein strongly labels the whole fungus, with no detectable effects on fungal growth and pathogenicity. Use of GFP to monitor survival and distribution was essential because: a) there were no precedents for the release of such fungal products, and b) there is an inherent paucity of knowledge concerning the fate of fungal genotypes at the population and ecosystem level. This ignorance has helped stir controversy concerning the risks and benefits of releasing transgenic (or foreign) fungi for disease control, insect, and plant pest management or bioremediation, and provides a powerful motivation for studies on their ecology. The field test confirmed that GFP is a very convenient way to monitor pathogen strains in field populations and demonstrated short term effects of insect transmission (non-target insects).

The most interesting result of the field trial was that it documented rhizosphere competence of an entomopathogenic fungus. This emphasizes that for many economically important pathogens the most understudied aspect of their biology involves the extended periods they survive in soil in the absence of a suitable host. Such knowledge is clearly of crucial importance for being able to predict and control outbreaks of plant or animal disease. Rhizosphere competence could be considered as a feature for selecting fungal strains for biocontrol. This would dovetail with attempts in 1PM to manipulate the environment of the plant and insect to enhance insect biocontrol. However, there are many environmental and economic reasons why researchers and industry would not seek to permanently establish an engineered microbial agent in the environment. Rhizosphere

competence might increase the difficulty of eliminating the pathogen following unanticipated and deleterious environmental effects.

Unfortunately, the current predictive data base for risk assessment issues regarding future releases of genetically engineered fungi remains small and very little is known concerning the survival of individual genotypes in the field. We still need to identify the lifestyle (saprotrophy or pathogenicity) responsible for maintaining the large populations of insect pathogens in soil. We also need to provide the knowledge required to predict and improve fungal responses to various environmental stimuli. In particular, to determine side-effects of genetic alterations on the survival of transgenics in soil, their interactions with other soil organisms, transmission to insects and genetic stability. Such knowledge might facilitate genetically based containment by reducing the ability of the organism to spread through a lack of saprophytic competence. I will discuss a recently initiated BRAG funded field trial that will address these issues.

Recently we have supplemented toxic proteins from the generalist *M anisopliae* strain 2575 with the insect-selective 70 an AaIT neurotoxin from the scorpion *Androctonus australis.* This toxin has already provided promising recombinant baculovruses, These studies are providing an opportunity to diversify the deployment of this useful, very well studied toxin, which like M. *anisopliae* has already passed many regulatory hurdles. Our results indicate that fungal and arthropod toxins have good killing power singly, but synergistic effects derived from combining them in a single strain produce a large magnitude of hypervirulence. One of our principal candidates for genetic enhancement is *M anisopliae sf acridum.* Its development as a locust mycoinsecticide is being hindered by its slow speed of kill. Strain 324 does not express several lytic enzymes/toxins produced by strain 2575, including phospholipases. Thus, we are investigating the extent of increases in virulence that result from appropriate combinations of several genes from *M anisopliae* strain 2575 encoding enzymes and toxins that act additively or synergistically to quickly kill insects or to prevent them from feeding. To analyze gene interactions, and the comparative efficacy of the AaIT with flingal toxins, we are comparing disease development (particularly speed of kill) by *acridum* transformed with two or more transgenes with equivalent *acridum* strains transformed with the Pri subtilisin gene or AaIT separately. Changes to LT_{50} values indicate faster kill consistent with toxicosis, while reductions in the median lethal dose (LC_{50}) values indicate that inoculum loads and efficiency of infection (attachment and penetration) are improved. We are also determining if any of the transformations broaden the conditions under which 324 or other strains can produce infection structures. Although we do not expect host range to change, we are evaluating the specificity of transgenic 324 against non-hosts compared with the wild-type (including *Apis mellifera, M sexta, Acheta domestica, D. melanogaster, Galleria mellonella* and *Tenebrio molitor).* The minimum dosage applied to an insect is 100-fold above the LC_{50} for the susceptible grasshopper host. By varying host density, relative humidity, and temperature, we are attempting to optimize the infection level within an insect population. Low infection rates using these procedures would probably translate into virtually undetectable infection rates under natural conditions.

CROSS-INFECTIVE MICROBES: FROM PLANTS TO HUMANS

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Microorganisms that infect and cause disease in both plants and people are uncommon but increasing in frequency of isolation. These cross-infective microorganisms are more insidious than those simply transmitted to humans by contact or consumption of plants. Currently 22 bacterial taxa and 38 fungal taxa have been reported as causing 'phytoses'. Several examples of bacterial and fungal diseases of plants and corresponding human disease will be presented. Questions that arise include accuracy of systematics analyses, role and similarity of virulence factors, genomic and pathogenicity islands and antimicrobial resistance. Newer biological techniques such as synthetic biology, illustrated by the construction of new viruses and DNA shuffling or intragenomic reconstruction, complicate oversight and regulatory action. Regulatory challenges among presumed equivalent taxa among plant and medical communities include definition and assessment of risk groups, permitting for interstate transport and differential perspective on the use and formulation of regulatory agency guidance documents. Assessment of alternatives for microbial pesticide niche markets will be presented. Potential interagency programs on cross-over pathogens will be discussed. The major challenge for agencies with regulatory responsibility for microbial biopesticides is the assessment and accuracy of taxa and scope of both natural and modified biological variations that may be used and their genomic stability. Management of cross-infective diseases of both plants and animals will require more interdisciplinary research and cooperative agency interactions.

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THE PATHOGEN/COMMENSAL PARADIGM; CAN TAXONOMY PREDICT RISK?

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The ability to determine the risk of contracting a food borne illness is dependent upon the ability to detect and quantitate the pathogen in the environment. Risk is the probability of an exposure to a specific hazard to cause harm. Hazard, then is the potential of the organism to cause harm, and under this definition, all strains of an organism do not represent the same hazard or the same risk. The use of conventional culture based methods to enumerate and identify pathogens has tremendous limitations in this capacity. These limitations can be overcome using molecular ecology techniques based on sequence comparisons of nucleic acids (DNA and RNA) and can be used to provide a molecular characterization, which can be used to predict virulence determinants and phylogenetic relationships. The application of a variety of nucleic acid probes and the polymerase chain reaction (PCR) can potentially provide a complete description of an organism's genetic composition, the extent to which these activities are expressed, as well as taxonomic information. Thus this molecular approach serves to evaluate the presence of specific sequences in the environment and provide a link between knowledge obtained in pure culture and the microbial populations they represent in the environment. Three enteric community examples are presented for which genomics based approaches have been used to address genetic relationships and disease or risk associations. First, is the example of the commensal-turned-pathogen, vancomycin-resistant *Enterococcus faecalis* described by Paulsen et al. (2003). *E. faecalis* is a prominent member of the commensal gastrointestinal microbiome. However, a subgroup of *E. faecalis* isolates harbor a virulence island that contains vancomycin-resistance genes a well as genes involved in virulence and infectivity. These *E. faecalis* isolates represent a hazard and as such detection and quantitation of risk must be specific for this genotype and not others that lack the virulence island. A second example relates to the enteric pathogen *Salmonella*, for which there are over 2,200 serovars. The majority of disease causing isolates (for warm-blooded animals) fall into subspecies I, which also represents more that 60% of all *Salmonella* strains identified. It is also clear that genotype and serotype are not congruent (Weigel et al., 2004). Therefore, as with *E. faecalis*, the target for predicting risk or hazard must be pathogen specific, not organism specific. The invA gene is the target of many of these methods because it not only is specific to the *Salmonella* genus, but it is also found in all known serovars of *Salmonella* (Chiu and Ou, 1996; Galan et al., 1992; Rahn et al., 1992; Swamy et al, 1996). However, even though this is a specific target, when Salmonella prevalence is low in a sample set, a pooled sample PCR may be needed as an indicator of the samples that are likely to be Salmonella culture-positive and reduce the amount of work associated with those samples likely to be culture-negative (Singer et al., 2006). Finally, *Escherichia coli* O157:H7 has been analyzed by octomer-based genome scanning which has demonstrated that there are two genetically distinct O157:H7 populations, one that causes illness and another that may be incapable of causing illness in humans or that is not easily

transmitted to humans from cattle (Kim et al., 1999, 2001). This group has also identified more than 100 genetic markers for the different populations some that may be used as markers in fast, simple tests to identify different O157:H7 populations in samples from humans and animals (Yang et al., 2004). Again this illustrates the paradigm of strains that are commensal and not pathogenic and those that are pathogens. Thus development of markers for risk must address the hazard and not merely the taxonomy of a microbial group.

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Regulatory Review of *Burkholderia cepacia* Under FIFRA

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The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) defines pesticides broadly to include agents, chemical and biological, which are intended to prevent, destroy, repel, or otherwise mitigate a pest. This definition includes bacteria which target other microbes and nematodes to preclude or antagonize the plant pathological properties of these infective agents. Hence, strains of the naturally occurring biological control organism, *Burkholderia* (*Pseudomonas*) *cepacia* and its related genomovars, were considered under the authority of FIFRA as part of the regulatory approval process for Section 3 pesticide registrations in 1992 and 1996.

During the course of the review process conducted by the Environmental Protection Agency (EPA), the taxonomic designation of the bacterium initially referred to as *Pseudomonas cepacia* was altered to reflect a greater understanding of its underlying phylogeny and an appreciation for the plasticity of the genome. *Ps. cepacia* was moved in 1992 into the newly established genus *Burkholderia* as *B. cepacia*, with recognition of its original isolation by Cornell University scientist Dr. Walter H. Burkholder in 1949 from diseased onions. As molecular techniques were refined, it became clear that the genome of *B. cepacia* consisted of 2 to 4 self replicating chromosomes and varied in size from roughly a size comparable to *E. coli* (4.5 Mb) to more than twice that. The taxonomic distinctions between species within this group were less than clear at the time the EPA was considering individual strains of this complex for registration as biopesticidal agents. The designation of *B. cepacia* for the purpose of this discussion will consider the species *B. cepacia* and related genetic species (genomovars; *e.g.*, *B. multivorans*, *B. vietnamiensis*, *B. ambifaria*, etc…), most of which have now received specific epithets as named species, as the '*B. cepacia* complex' or simply '*B. cepacia* '.

The guideline studies which must be addressed as part of the FIFRA Section 3 registration process at EPA include those concerned with human health issues and a second set of standardized tests aimed at uncovering any environmental concerns. The human health tests include intravenous, oral, pulmonary, and ocular challenges, using a rodent model and a dermal toxicity / pathogenicity test using a rabbit model. The results of these tests with the *B. cepacia* isolates submitted for review in 1990 by Stine Microbial Products were largely unremarkable (no experimental animal deaths resulted). Similarly, the environmental / non-target organism guideline pathology / toxicity tests, including avian oral challenge, freshwater fish, beneficial insects, honeybees, earthworm, and non-target plant studies were without evidence of significant toxicity or pathogenicity for the test organisms.

A FIFRA mandated Science Advisory Panel (SAP) was convened July 20 – 23, 1999, to discuss the fate of the *B. cepacia*-based fungicidal products (*i.e.*, Blue Circle, Deny) as registered in 1996 by Stine Microbial Products. A growing concern in the medical community indicated that *B. cepacia* (the complex) was capable of colonizing patients afflicted with cystic fibrosis and chronic granulomatous disease. A significant decrease in lifespan of individuals colonized with *B. cepacia* was noted in some instances, although of the total number of Cystic Fibrosis (CF) patients in the United States, approximately 3.5 % were known to be colonized with *B. cepacia*. The colonization and changes in health status attributed to this bacterium became known as 'cepacia syndrome' and had serious health and practical implications for CF patients who were no longer allowed to join Cystic Fibrosis Foundation (CFF) functions due to fear of patient to patient spread.

While it is clear from the FIFRA SAP report (1999) that taxonomic distinction of the various genomovars of *B. cepacia* would not serve adequately to allow for 'safe' biopesticidal strains to be distinguished from those with clinical or nosocomial potential, the question of possible human exposure to *B. cepacia* applied as a seed treatment, soil drench or side dress treatment was less than certain. The SAP report detailed what was known about the levels of *B. cepacia* occurring naturally in agricultural fields with various crop plants and compared this with what levels would be anticipated given application of the highest label rates of Deny® Fungicide. This rate would result in the approximate addition of 3.7×10^{11} cfu/ha, while the available measurements of naturally occurring *B. cepacia* suggest a background population 7.5×10^{12} cfu/ha. The net result is an anticipated transient increase in *B. cepacia* population size of approximately 5 %.

It is notable that the CFF at the time of the SAP and also currently on their website through associated videos does not request that CF patients refrain from gardening, contact with soil, plants, raw vegetables or salad bars. If the environment is seen as a harbor for *B. cepacia* strains with potential to develop into clinical pathogens or as a source of virulence genes which may be exchanged with human-associated isolates, then exposure to these sources should be considered as significant. Further, it is noteworthy that the primary use of the Deny® Fungicide product containing *B. cepacia* was as a seed treatment in which seeds of peas, maize, soybeans and other crops would be treated prior to planting and placed into the soil using mechanical seed drills and covered. The route of increased exposure to humans, other than applicators, from this delivery system remains nebulous and even doubtful, however, it was not fully explored in the SAP analysis due to a variety of factors. As a result of a subsequent 'data call-in', Stine Microbials was requested to conduct several field tests to determine the potential for aerial transport and survival of their *B. cepacia* strains in the environment, as well as development of more detailed genetic information. In 2000, the company decided to voluntarily cancel their registration and limit distribution of remaining stocks.

The risk assessment of *B. cepacia* and, more importantly, the political ramifications of the species' unfortunate association with a serious human disease condition, dictated that the EPA must act to resolve the contentious issue of *B. cepacia* and human health risk. Unfortunately, the emphasis on the 'hazard' side of the risk equation (*i.e.*, clinical infection) overwhelmed the 'exposure' (*i.e.*, increased human exposure in agricultural

settings) side of the equation during the SAP process. With a two thirds panel membership consisting of medical / clinical personnel, it is noteworthy in the panel report that the 'majority' opinion of the SAP appears to dismiss the lack of increased environmental exposure from biopesticidal applications. Given the lack of demonstrable clinical infection of CF patients from environmental *B. cepacia* isolates and the decrease in numbers of CF patients infected with *B. cepacia* since institution of measures to reduce patient contact with cepacia positive individuals (*e.g.*, elimination of CF summer camps, segregated clinics), it would appear that patient-to-patient spread of this organism is certainly worthy of attention, but perhaps environmental exposure and outcome are weighted too heavily in the absence of precise data.

The take home message from this retrospective look at the history of *B. cepacia* as a biopesticidal agent is that meeting early on with the regulators who will be assessing the agent in question may save some significant time and expense. Even then, however, as new scientific information related to the organism comes to light, the risk assessment process may take on renewed life with different resultant conclusions.

[http://www.epa.gov/scipoly/sap/meetings/1999/index.htm#072099] - FIFRA authorized Science Advisory Panel

[http://www.epa.gov/pesticides/biopesticides/regtools/guidelines/40cfr158_740c.htm] – EPA Human Health Testing Guidelines

[http://www.epa.gov/pesticides/biopesticides/regtools/guidelines/40cfr158_740d.htm] – EPA Non-target Organism Testing Guidelines

Risk Assessment and Biotechnology – 'Get Jiggy with it!'

Charles Yoe

Biotechnology issues are of global importance and affect billions of people. They involve a great variety of wickedly complex problems. The solution of these problems is not well served by traditional planning and analytical methods because the world is uncertain and uncertainty means risk. Traditional food safety systems are no longer enough. Experience shows science-based approaches to our problems have helped. Risk analysis is a relatively new way of approaching problems that integrates science and social values. Risk analysis is a paradigm shift, a way of approaching problems. It comprises the three tasks of risk management, risk assessment and risk communication. It is not science but it is science-based and there are compelling reasons for doing risk analysis. It may be useful for managing biopesticides and transgenic insects.