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Review article

Symbiotic Control in agriculture and medicine

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Abstract

New methods are being reported that offer unprecedented opportunities to address pest and disease problems in agriculture and medicine. One of the most exciting areas of application employs symbiotic organisms to deliver anti-disease or other strategies. The new methods sometimes use recombinant methods to produce genetically modified microbes. The novelty of employing recombinant organisms to address traditional and here-to-fore insoluble problems finds the regulatory community lacking precedents for how to proceed.

Keywords: Paratransgenesis, Chagas disease, Pierce's disease, glassy-winged sharpshooter, aflatoxin, dental caries, regulation, competitive displacement

1. Introduction

Paratransgenesis

A new genetic transformation process, called paratransgenesis, was developed by Professor Frank Richards at Yale University and colleagues (Beard et al., 1998, 2001, 2002; Rio et al., 2004) for use in preventing the transmission of pathogens by insect vectors to humans. Paratransgenesis in this application means genetic alteration of symbiotic microbes that are carried by insects (therefore, they are paratransgenic insects). This overall strategy of disease prevention is called Symbiotic Control and is a variation on the theme of symbiotic therapy (Ahmed, 2003).

Symbiotic Control

The technique of paratransgenesis was employed to create conditions that render insects vector-incompetent. The strategy of Symbiotic Control employs both paratransgenic and non-recombinant methods to control disease or health problems. In some cases these solutions may entail simple competitive displacement. Genetic manipulation has fitness costs that must be factored in to the application.

The key to Symbiotic Control is finding a candidate microbe having an existing association with the ecosystem that includes the problem or condition at hand and that occupies the same niche as or has access to the target pathogen or condition. Symbiotic Control differs from classical biological control, where foreign herbivores, parasites or predators are sought abroad for importation and establishment in a local ecosystem to control a pest such as a plant or invertebrate. In Symbiotic Control, all elements are at the local site and established in the ecosystem; foreign exploration is not only unnecessary, but also most likely counter-productive. Because of these strict requirements, a suitable symbiotic candidate may not always be found or may not be amenable to practical manipulation.

Once a candidate symbiont is identified as a delivery vehicle, all manipulations can be local. Indeed, a Symbiotic Control solution developed for one specific location may not be suitable for another site or condition elsewhere.

Once a microbe is identified as a vehicle for Symbiotic Control, it is studied for ability to culture and re-release and suitability for genetic alteration, if necessary. Beyond that a method of re-introduction of the symbiont is needed. The methods selected have to be adaptable to ordinary practices in the target area. Finally the microbes chosen for Symbiotic Control must pass regulatory scrutiny.

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Fitness of genetically modified microbes

If a candidate microbe is selected, cultured and genetically modified, and reintroduced, it is displaced eventually by native counterparts already present. Genetic manipulation exacts to transgenic organisms a fitness cost (Catteruccia et al., 2003; Silbermann and Tatar, 2000; Purrington and Bergelson, 1997). All other aspects of the Symbiotic Control organism are the same; indeed, this approach would not function properly if the symbiont were not recognized as virtually the same organism competing in the natural environment. This includes ability to interact with host organisms.

One aspect of this approach is that the genetically altered organism is less fit than the unmodified counterpart. On the other hand, this is one aspect of the strategy offering regulatory assurance that the life of the introduced organism is limited.

Crop and health protection methods

The tools for agriculture and health protection can be categorized as cultural, chemical, biological or strain improvement (Van Emden, 1989). When a new pathogen appears, the immediate response is isolation and removal of infected plants or animals and measures to interfere with the mode of transmission. This approach is used with bird flu in Europe, Asia and elsewhere, Pierce's disease in grapevines in California, Citrus Canker in Florida, Ring Spot Virus on papaya in Hawaii and Citrus Variegated Chlorosis in Brazil.

Table 1. Classic crop and health protection methods.

Method	Agriculture	Medical
Mechanical	Cultivation	Sanitation
Chemical	Pesticide	Antibiotic
Biological	Biological control	Probiotic therapy
Genetics	Resistant varieties	Improved breeds

The second stage of response is chemical if a disease pathogen or pest is carried by vector insects that can be treated with insecticides. Curly Top Virus (CTV) Control program in California involves treating the over wintering sites of the only vector, beet leafhopper, *Circulifer tenellus* (Homoptera: Cicadellidae) with malathion insecticide along with an extensive monitoring program. This program has been in operation since 1943 and is paid for by a box tax on the affected commodities. All evidence points to success of this program. Elsewhere, periodic outbreaks of CTV in the western United States cause sporadic losses in sugar beets, tomatoes, peppers and melons, the susceptible crops.

Chemical treatments act as the first line of defense, but are unsatisfactory in the longer term. More sophisticated approaches take much longer to mobilize, such as development of pest or disease resistant varieties of plants or animals, or establishment of biological or cultural control paradigms to bring a pest under control. These more sustainable methods can include simple choices such as crop rotation or sterilization measures.

Classical biological control as used in crop protection depends on a natural enemy or pathogen that attacks a pest insect or weed. Usually this requires discovery of the home origin of the pest organism, which is thought to contain the richest complex of parasites and predators that have evolved with the pest organism over the longest period of time. A pest kept naturally to low numbers in the area of origin should be a good source of biological control organisms for importation and trial locally (Torchin et al., 2003).

The philosophy of Symbiotic Control

In contrast to the crop and health protection methods mentioned above, Symbiotic Control does not aim to control the vector of a condition or disease. Rather, it is designed to affect the pathogen, either directly by affecting its ability to survive or indirectly by affecting its ability to be transmitted by the vector. In most cases this is achieved through competitive displacement of a microbial pathogen with a symbiotic microbe. Symbiotic Control can be extremely selective, lacking side-effects.

All of the disease examples used above have no cures; indeed, there are no cures for plant diseases caused by pathogens carried by insects. Symbiotic Control offers a new strategy. Several examples given below illustrate this approach.

2. Examples of Symbiotic Control

The first two examples of Symbiotic Control described below are the most advanced in terms of regulatory approval.

Aflatoxin

Aflatoxins are natural metabolites (mycotoxins) of several species of the fungus, *Aspergillus flavus*. The fungi reside in soil but can colonize crops if moisture conditions are suitable. Aflatoxin outbreaks are often associated with drought conditions. Aflatoxins can contaminate cereals, oil seed crops and nut crops. All animals are susceptible to aflatoxin poisoning that can lead to liver damage and induce liver cancer under certain conditions. Although humans have a fairly high tolerance (Williams et al., 2004), the presence of aflatoxins in several crops such as cotton

seed meant for animal feed or peanuts harvested for human consumption can trigger seizure of the commodity with severe economic consequences to the grower.

Peter Cotty of the USDA-ARS and his colleagues developed a Symbiotic Control strategy to prevent contamination of cotton seed by aflatoxins for the Arizona cotton growers. Several strains of *Aspergillus flavus*, the most common source of aflatoxin contamination (Cotty et al., 1994), were screened and tested for production of aflatoxin. One of these strains, AF-36 (Ehrlich and Cotty, 2004) was found not to produce aflatoxin (atoxigenic) and was recently registered as a biopesticide with the US Environmental Protection Agency for treatment of soil prior to cotton growing (Antilla and Cotty, 2002; Cleveland et al., 2003; Jones, 2003).

When cultivated on wheat seed remnants and broadcast dry onto soil, AF-36 displaces other *Aspergillus* species leaving the subsequent cotton crop relatively free of aflatoxin contamination in a classic competitive displacement response. Treatment of a single field has a ripple effect, spreading the fungus to adjacent fields and lasting more than one year in the treated field. Each yearly treatment affords greater protection. The Arizona Cotton Growers Association produces AF-36 in hoppers at their Phoenix, AZ facilities and charges $5.00 an acre for the treatment of cotton fields in Arizona.

Georgia peanut growers have duplicated this effort and have their own version of AF-36 used to treat soil prior to planting peanuts.

[http://archives.foodsafetynetwork.ca/agnet/2004/6-2004/agnet_june_22-2.htm#story3]. This version of AF-36 was registered separately as Afla-Guard® (Hagan, 2005) and is reported to reduce aflatoxin contamination by 60–98 percent.

Dental caries

A recent innovative control of dental caries (Hillman, 2002) has been described that employs a competitive displacement strategy of Symbiotic Control. A strain of *Streptococcus mutans*, a common mouth bacterium of humans, was selected that produces a natural mild antibiotic presumably that aids in displacement of other bacteria. The genes responsible for producing enzymes responsible for converting glucose to lactic acid were then removed by recombinant means.

Oragenics, Inc was founded to develop *S. mutans* as a treatment against tooth decay. The resulting product uses what Oragenics calls replacement therapy [www.oragenics.com] and is at the leading edge of a new vision of oral biology (Pennisi, 2005). As a potential commercial product using a recombinant bacterium for preventing tooth decay, it is pioneering Symbiotic Control of the modern type.

The Oragenics Company received a US Food and Drug Administration permit for field safety trials of replacement therapy using recombinant *S. mutans*, on 30 November 2004. The first year of safety trials is being followed by a second year of field tests using human volunteers.

The aflatoxin case described in the previous section and dental caries protection both use microbes in Symbiotic Control. Both examples could also be called replacement therapy since the underlying principle is competitive displacement. The atoxigenic strain of *Aspergillus flavus* in AF-36 lacks a gene that supplies one of the reaction steps in biosynthesis of natural aflatoxins. Although this mutation was found by screening natural populations from soils in Arizona, it could also have been produced by recombinant methods. If so, the *A. flavus* of AF-36 would be analogous to the *S. mutans* of Oragenics.

Symbiotic Control of medical conditions

Inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's Disease each affecting different parts of the bowel. Although the cause of IBD is not known, the symptoms are caused by inflammation of the mucous lining of the bowel. The condition affects people 15–35 years of age, most often in Western Europe and North America.

Treatment for IBD involves both anti-inflammatory medication to interrupt the inflammatory tissue reaction and immuno-repressive therapy. Symbiotic Control of IBD was reported when strains of gut-compatible bacteria were recruited to deliver anti-inflammatory agents, such as interleukin-10 (Westendorf et al., 2005).

Other applications of Symbiotic Control include protection against HIV (strategies for preventing HIV infections via symbiotic bacterial delivery (Chang et al., 2003; Rao et al., 2005). A probiotic strain of *Escherichia coli* (Nissle 1917) was used to deliver HIV-gp41-hemolysin A, hybrid peptides that block HIV fusion and entry into target cells. Micromolar amounts of the protective peptides were reported and the Nissle 1917 strain of genetically engineered bacteria colonized mice for periods of weeks to months. The colonies were present in the rectum, vagina and small intestine, or at or near the site of contact with the virus.

In a commentary to the Rao et al. (2005) article mentioned above, Lagenaur and Berger (2005), emphasized that this approach using a live bacterium, while not new by itself, is novel in the ability to genetically engineer into the delivery vehicle superior therapeutic gene products. Also, while a strictly "competitive displacement" action is not being conducted, this "microbicide" (what the Regulatory Agencies would call a biopesticide) agent would be considered a therapy rather than a control as it passively awaits the possibility of viruses arriving. Lagenaur and Berger also point out that it would be far less expensive and far more convenient to maintain a therapeutic agent in a live bacterium instead of on a shelf in inanimate storage.

Other possibilities of delivering solutions to human afflictions are being described on a regular basis. Delivery of anti-cancer therapies in humans via genetically altered or other symbiotic bacteria (Pilcher, 2004) is one of them.

Chagas disease

The original example of Symbiotic Control remains the Chagas disease application (Durvasula et al., 1997, 1999a, 1999b; Beard et al., 1998, 2002), which originated in the laboratory of Professor Frank Richards at Yale Medical School. Chagas disease is caused by a protozoan pathogen, *Trypanosoma cruzi* that is carried in the hind gut of blood-sucking bugs of the triatomine group, also known as cone-nose bugs or kissing bugs. Following a blood meal, usually taken at night during sleep, the triatomine insects process the blood meal rapidly and defecate near the feeding site. The deposits are contaminated with the pathogen that can inoculate the human victim when scratched.

A Symbiotic Control candidate was isolated by Ravi Durvasula (Durvasula et al., 1999a) from the symbionts occupying the same niche in the hindgut as the pathogen. *Rhodococcus rhodnii* was isolated from *Rhodnius prolixus*, one of the triatomine vectors of *T. cruzi*. Initially, the antibacterial peptide, cecropin A, was expressed in the Chagas vector at levels that eliminated the parasite. Subsequently, a single chain antibody fragment was expressed and the Durvasula lab is currently expressing single chain antibodies that target key epitopes of *T. cruzi*.

Pierce's disease

Pierce's disease (PD) in grapevines is caused by the bacterium, *Xylella fastidiosa*, which blocks the xylem flow of affected plants (Hackett et al., 2003; Hopkins and Purcell, 2002). Various strains of *X. fastidiosa* each are associated with particular host plants (Hendson et al., 2001; Rodrigues et al., 2003; Schaad et al., 2004; Hoddle, 2004). Disease symptoms are produced in only a few of the plants infected by any strain of *X. fastidiosa* (Purcell, 1997). In transmission experiments, a strain of *X. fastidiosa* isolated from oleander did not cause disease in either grapes or almonds, although the bacterium was present in all of these plants (Almeida and Purcell, 2003).

The *X. fastidiosa* strain causing PD in grapevines in California (PD-XF) probably came from native origins in the temperate band running from Florida through Texas to northeastern Mexico (Hoddle, 2004). PD-XF has been in California for many decades (first outbreaks recorded in 1883), but previously appeared infrequently because native sharpshooters that act as vectors only occasionally attempt to feed on grapevines (Anonymous, 2002).

This situation changed dramatically with the arrival of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Redak et al., 2004). [The name *H. coagulata* was recently changed to *H. vitripennis* (Takiya et al., 2006)]. The difference between GWSS and native leafhoppers is the difference between a minor nuisance and a major epidemic in terms of appearance of PD and related diseases. Before the arrival of GWSS, PD was handled as a short-term problem. With the arrival of GWSS, PD became an emergency that threatens large areas wherever GWSS becomes established and is being addressed statewide with quarantine restrictions on movement of host plant materials from nurseries.

In addition to PD, other strains of *X. fastidiosa* have been transmitted by the GWSS to other host plants such as oleander, and liquid amber, in which *X. fastidiosa* causes disease. Oleanders are used extensively as ornamentals on California freeways or along the sides of right-of-ways. Loss and replacement cost of oleanders on highways in California was estimated at $125 million.

PD outbreaks in vineyards in Temecula and Bakersfield have been checked recently by the systematic insecticide treatment of winter resting sites used by adults. This and spot treatments have decreased spring populations to levels that decrease the PD threat, and slowed the spread of GWSS north through the San Joaquin Valley. Citrus is a preferred habitat for GWSS in California. Vineyards are at greatest risk when they are located near citrus because of movement by GWSS in the spring (Blua et al., 2001). However, field entomologists readily admit these measures are short-term and a longer term solution is needed.

The objective or rationale for developing a method of Symbiotic Control for Pierce's disease is to disrupt vector transmission with the least effect on other crops. Symbiotic Control would be available to local vineyards for local control instead of area-wide treatments of alternative host plants such as is done now. Treatment of citrus with systemic insecticides for GWSS to reduce the chance of acquiring and spreading pathogens in adjacent vineyards cannot be seen as a long-term solution. Symbiotic Control would be more selective and have the least side-effect on other biological control practices. The Symbiotic Control organisms inhabit the xylem fluid of the target plants yet do not contaminate the berries of the grapevines. It remains to be seen if one treatment would be effective for an entire season.

Symbiotic Control of Pierce's disease

Three potential bacterial candidates for Symbiotic Control were collected from GWSS in southern California (Bextine et al., 2004). All were endophytes transmitted to different host plants by GWSS in a manner analogous to the pathogen; thus, the candidates had access to the pathogen in host plants or in the insect vector. This provided the needed properties for access. *Alcaligenes denitrificans* var. *xylosoxidans* (*Axd*) was selected for further development.

Using methods perfected in previous studies (Lampe et

al., 1999, 2000) *Axd* was genetically altered to contain a *Ds*Red fluorescent marker gene (Bextine, et al., 2004). The marker gene was inserted into the chromosome, not a plasmid in the bacterium. *Ds*Red *Axd* was found to be transmitted by GWSS and to colonize various plants (Bextine et al., 2004, 2005). *Ds*Red *Axd* could be introduced into grapevines by misting the leaves or by soil drench or by direct injection of the stem of the grapevine. *Axd* appeared to be far more adaptable to citrus than to grapevine (Bextine, et al., 2005). Indeed, the original samples of GWSS from southern California were obtained from citrus groves in the Agricultural Operations plots at the University of California, Riverside, so it is likely that the endophytes in GWSS came from citrus.

A number of candidate antimicrobial peptides were screened for possible use in Symbiotic Control of Pierce's disease (Kuzina et al., 2006). Dave Lampe further screened single chain antibodies from a phage antibody library for ability to bind the coat protein of the pathogen, *X. fastidiosa*. He selected antibody that was specific for the strain of *X. fastidiosa* causing Pierce's disease and did not recognize closely related *X. fastidiosa* strains, the S1 antibody.

A disease cycle protocol was developed (Bextine and Miller, 2005) in which *X. fastidiosa* was infused into 5 cm sections of cut chrysanthemum stems. Sharpshooters allowed to feed from these *X. fastidiosa*-stems were able to transmit *X. fastidiosa* to about 50% of subsequent chrysanthemum stems used as clean recipients. This efficiency of transmission matches the known less efficient transmission of *X. fastidiosa* by GWSS compared to native leafhoppers in California. When the *Xylella*-GWSS fed on chrysanthemum stems infused with the S1 phage antibody in suspension, the vector insects lost the ability to transmit *X. fastidiosa* to the recipient stems. It was unclear from these preliminary results how the transmission was disrupted.

3. Regulatory Activities

Field testing of recombinant Alcaligenes

When the marked *Axd* was available, we applied for permits to do field testing (Miller, 2004). The request was sufficiently novel that a regulator agency with jurisdiction was not readily identifiable. Finally USDA-APHIS-BRS (United States Department of Agriculture, Animal Plant Health Inspection Service, Biotechnology Regulatory Service) declined to review and EPA accepted the application. The EPA found the applicant organism [was not in a clearly defined jurisdiction]. If *Axd* made a gene product that controlled a pathogen, it would be called a microbial pesticide and would be regulated by the BioPesticides and Pollution Prevention Division (BPPD); however, with just a marker gene inserted, *Ds*Red *Axd* did not fit any existing regulatory structure and therefore was defaulted to the Office of Pollution Prevention and Toxics (TSCA) Biotechnology Program.

It took longer than the statutory one month period for TSCA to evaluate and issue a permit for field testing. Field tests of *Ds*Red *Axd* were approved for injection into grapevines in commercial vineyards in four places in California, three in southern California in the endemic GWSS area and one in wine-growing area of Napa Valley well north of the GWSS-infested area. As a condition, the agency required that all test material be destroyed following the tests and the soil around the root ball had to be sterilized. We originally proposed to cover the test plants with gauze to prevent insect access to the test grapevines.

Several things about this permit are worth noting. Despite the stringent conditions placed on the vineyard sites, the University of California, Riverside BioSafety Committee approved use of the exact same organism at the BL-1 level. Organisms in this category can be used in High School Biology Laboratories. Thus exposure of students to *Ds*Red *Axd* was acceptable, but injecting into grapevines outside the laboratory was not.

Regulation was responsible for stopping development of another bacterium for agricultural use. *Burkholderia cepacia* had been sought as a biological control agent for its ability to prevent leaf and stem blight, root rot and *Pythium* diseases of cucumber and peas (Holmes et al., 1998). Reports of nosocomial infections of *B. cepacia* in lungs of cystic fibrosis patents in part blocked approval of registration. Widespread resistance in *B. cepacia* to antibiotics and differences of opinion about the threat this represents will probably prevent development for use in agriculture in the near term.

Progress made to date includes development of methods of inserting genes into symbiotic bacteria as vehicles of Symbiotic Control of Pierce's disease. While a candidate endophyte was identified (Parker, 2006), it is a citrus endophyte and other endophytes have been isolated from grapevines that are more suitable for use in vineyards. Lethal genes have been identified and methods have been perfected for insertion into endophytes (Lampe, 2006). Permits were obtained for field trials using genetically marked endophytes. The genetically modified endophytes did not persist more that four weeks in grapevines in commercial vineyards. The endophytes were not toxic to insects and arthropod predators even when injected at excessive doses. The approach can be applied to a wide variety of plant diseases caused by pathogens that are transmitted by insects.

Regulatory guidelines are currently being drafted to address issues of risk assessment and permit and registration procedures. Based on similar guidelines for response to global warming, the Kyoto Protocol, these for transgenic, paratransgenic and biopesticide tools are called

"Daegu Protocol" after the site of the meeting, International Congress of Insect Biotechnology and Industry, scheduled for 19–24 August 2007 in Daegu, Korea. The activities surrounding the Daegu Protocol will be posted at the website: http://biopesticide.ucr.edu, that was used for a Workshop on improving communication between the regulatory and scientific communities in the United States.

4. Summary

A new method, Symbiotic Control, was described to address pest and disease problems in agriculture and medicine. The advantage of the new method is extreme selectivity and applicability to local conditions. The main disadvantages are lack of a broad market and economic drive to force a solution. While the application is new to the regulatory process, existing law can be applied and protocols are in place to assure safe trials. Time will be needed to approve trials because the approach is new and untested. A number of examples were given of the application of Symbiotic Control, which operates on a principle of competitive displacement. Time and funding are needed to address ecological and side-effect issues associated with these applications.

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