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INVITED REVIEW

Pest and disease challenges and insect biotechnology solutions*

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Abstract

Advances in microbiology and molecular genetics have led to renewed interest in microbial and host interactions, especially mutualism and symbiosis. More genome sequences are being reported every year; indeed, we are awash in information on an unprecedented scale. However, despite the greater amount of genomic information, we still have difficulty resolving species boundaries, and we still have much to learn about pathogen, vector and host interactions. Biotechnology approaches offer the promise of new tools for pest and disease control.

Key words: biotechnology, genomics, symbiosis.

Introduction

The Korean Association of Biological Sciences prepared a special symposium for the "Biological Year of 2007." In the spirit of biology in the year 2007, one of the newest societies in the biological sciences, the International Symbiosis Society (ISS; http://people.bu.edu/iss), operated by President Douglas Zook out of Boston University, MA, USA, is celebrating its tenth year. Founded in April 1997, the ISS has grown rapidly and has held its last two congresses in Halifax, Nova Scotia, Canada in August 2003 (the fourth) and Vienna, Austria in August 2006 (the fifth).

Studies of mutualistic relationships between organisms, including microbes and eukaryotes, reveal a rich complexity. Nature continues to surprise us. The theme in this review celebrates life by considering that increased complexity as revealed by modern molecular methods. Despite the increasing amount of information, we are still far short of understanding and converting information into applications. It is sobering to realize the hurdles to understanding the natural world of pests and diseases.

The "new biology"

In his book *The Ancestor's Tale*, Dawkins wrote "Creatures with complementary skills flourish in each other's presence . . . The entire genome . . . is an ecological community of genes that flourish in each other's presence" (Dawkins 2004, p. 490). This, it seems to me, illustrates the dynamics of symbiosis and how it is a fundamental part of all biology. Indeed, Dawkins reached the conclusion that higher life owes its beginnings and existence entirely to symbiosis and the merging of organisms.

Steven *et al*. (2006) reported that 100 trillion bacterial cells reside in the human gut. Long ignored because of an inability to culture the majority of these microbes, newer polymerase chain reaction (PCR) methods now allow identification. With this new information comes an overwhelming wave of complexity. We are not yet certain how to deal with the increased amount of information.

The first attempts to incorporate this vast amount of new information into biology led to the construction of a new taxonomic group of microbes, the Archaea (Miller & Day

2004a). This was an order of magnitude higher than the entomologists discovering an entire new Order of insects, the Mantophasmids, that was not known before (Klass *et al*. 2002) and whose members are alive and well in southwest Africa.

Xu and Gordon (2003) appreciate this new symbiotic complexity and suggest that microbes influence the development and function of the human gut, and that this suggests new ways of appreciating the foundations of health. At the same time, the company Oragenics (http://www.oragenics.com) uses microarray analyses to characterize the microbial floral of the human mouth. They use this information to diagnose gum disease and are beginning to develop treatment protocols based on the community of symbionts.

Because the field of symbiosis was moving ahead so rapidly, we developed a series of books of contributed chapters to try to keep abreast of these developments (Bourtzis & Miller 2003, 2006). The contributions from the *Wolbachia* field are abundant because of the preponderance of work being done there. *Wolbachia* is an intracellular "symbiont" that is transmitted vertically in the eggs. Although the *Wolbachia* story is by now familiar (*Influential Passengers*; O'Neill *et al*. 1997) until recently an advantage to having *Wolbachia* infection was not clear. Elizabeth McGraw (2006) reported last year that *Drosophila* females infected with *Wolbachia* laid more eggs in conditions of either iron deficiency or iron excess compared to uninfected females.

As I prepared this lecture in spring of 2007, the genome of the yellow fever mosquito is being reported. At this time the project director, David Severson, gave a talk at my campus, University of California Riverside, CA, USA, on what the project is finding. He reminded us that there is no cure for dengue hemorrhagic fever (DHF) and no vaccine; and that vector control (the genus *Aedes* is the vector) is the only current option to keep the disease at bay. I was struck by the vast amount of information now available on the *Aedes* web site.

The authorities responsible for insect genome projects have created a website (http://www.vectorbase.org) that provides an entrypoint to deal with the increasing information concerning arthropod vectors of disease pathogens.

Gene movements and "foreign" genes

A new topic in risk assessment of genetically modified organisms is horizontal gene movement. To put this into perspective, the first descriptions of the human genome uncovered the startling fact that upwards of half of the genome is made up of genetic elements coming from elsewhere (Luning & Kasazian 2000). We know these as mobile elements. We now realize that mobile elements are very common in genomes. In the tobacco budworm *Heliothis virescens*, *piggyBac* transposable elements are intact and extremely active (Wang *et al*. 2006) and *mariner*-like elements are intact as well (Ren *et al*. 2006). At any given time, the genomes of individuals in a given population are modified depending on where the elements insert. These elements are thought to play a role in evolution, and when they are active in nature, they could be responsible for the variability of natural populations.

In contrast to the active elements mentioned above, the genome of the pink bollworm *Pectinophora gossypiella* contains inactive *mariner*-like elements (Wang *et al*. 2005). The *piggyBac* transposable element originally isolated from the cell line of a cabbage looper moth, *Trichoplusia ni*, is commonly used in transformation experiments (Miller 2004; Dafa'alla *et al*. 2006), and this has led to stable strains that have been reared in quarantine through some 80 generations, from 1998 to the present (Miller 2004). The stability of elements inserted into pink bollworm within the *piggyBac* cassette correlates with a narrow host range of the insect and lack of flexibility and adaptability in nature. The pink bollworm is a pest only of high-yielding commercial cotton.

The tobacco budworm *H. virescens*, also known as the tomato fruitworm in America, has a wide host range and prodigious flying ability, and is also a perennial pest in different crops, from cotton to geraniums. Any given population of tobacco budworm has larvae exhibiting a multitude of colors and patterns. It now appears possible that active mobile elements known to be in the genome could explain the variability of the phenotypes and possibly the broad host range.

More investigation into this phenomenon needs to be done, but for a start, pest insects can not be viewed in the same way as before. They are now thought of as variable at the genome level. Again, one returns to the Dawkins quote "The entire genome . . . is an ecological community of genes that flourish in each other's presence" (Dawkins 2004, p. 490). Only now the genome structure of nucleotide sequences is viewed not as static as this, perhaps, implies, but as much more dynamic than previously realized. This makes sense when we appreciate the adaptability of the Heliothine complex.

Genes moving and organisms adapting are both much more common in bacteria than in other organisms (Pennisi 2003; Miller & Day 2004b). The phenomenon of plasmid transfer in bacteria is well known as a source of development of resistance to antibiotics, for example. With the acceptance of normal gene movement between organisms, one can appreciate what this means in terms of regulatory monitoring. When is gene movement "natural" (and therefore acceptable) and when is gene movement "artificial" (and perhaps to be regulated)? The world is currently coming to grips with this new reality.

Information overload

Insect biologists have only recently joined in the process of converting biotechnology into applications, while symbiosis

and symbionts are beginning to provide interdisciplinary ways of looking at medical and agricultural problems. In the meantime, biology is awash in new information. According to Juan Enriquez (2005), "The largest databases in the world are now biology databases. About half of this data is free. Most of it did not exist five years ago" and "Free public databases increased from 171 at the beginning of 2004 to 719 by January 2005."

It has become routine to deposit DNA sequences into GenBank. We have allowed genes to be "patented." The information explosion in biology that is inherent in terms such as metagenomics, epigenetics, proteomics and microarray technology is matched by technical advances in computer storage capacity and miniaturization. The increased information can be stored in increasingly smaller spaces. What will we do with all of this information?

Real solutions still evade us

One would think that with all of the new molecular biology information and all of the new information handling ability we should be able to solve some of our oldest pest and disease problems. The reality is different. California is invaded by a new pest or disease every 60 days. We have been able to eradicate only a few pest insects in very specific cases. Mostly, the new pests are here to stay.

The greater scientific ability has not led to new advances yet, but it has certainly allowed more detailed descriptions – down to the nucleotide sequence level. The taxonomists tell me, however, that we are still unable to bring focus and closure to given species or taxonomic relationships. We still keep changing the names of insects, which gives us the illusion that we understand them as pest problems.

The new biotechnology does provide new ways of looking at old problems. Paratransgenesis is offered as a strategy to prevent Chagas disease (Durvasula *et al*. 2003) and to control Pierce's disease of grapevines (Miller *et al*. 2006). *Wolbachia* are seen as a new type of "biological control" tool for population suppression (Bourtzis & Robinson 2006).

A host of other paratransgenic (or symbiotic control) projects are underway. Some of these are described at http://www.symbiosis.ucr.edu, where we maintain a webpage to keep track of them world wide. However, the regulatory activity associated with these projects is stifling their development. As mentioned above, it is becoming difficult for the public, and I include the scientific public in this, to accept these new methods.

AF-36 to control aflatoxin contamination

A few of the several strains of the common soil fungus *Aspergillus flavus*, and related species, produce aflatoxins as natural metabolites. These mycotoxins can contaminate cereals, oil seeds and nuts. Mammals are susceptible to aflatoxin poisoning. 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 and corn harvested for human consumption, can trigger seizure of the commodity and loss of income to the grower.

Peter Cotty and colleagues (Cotty *et al*. 1994) developed a treatment based on a competitive displacement strategy to prevent contamination of cotton seed by aflatoxins for the Arizona cotton growers. They tested several strains of *A. flavus* for production of aflatoxin. The thirty-sixth strain tested, AF-36 (Ehrlich & Cotty 2004), lacked the ability to produce aflatoxin (it was atoxigenic). AF-36 was registered as a biopesticide with the US Environmental Protection Agency, and is used today as a preplant soil treatment for cotton fields (Antilla & Cotty 2002; Cleveland *et al*. 2003; Jones 2003).

AF-36 displaces other *Aspergillus* species in the soil, leaving the subsequent cotton crop relatively free of aflatoxin contamination in a classic competitive displacement response. The fungus on treated fields lasts more than 1 year and spreads to adjacent fields, offering the same protection there. The project was funded by the Arizona Cotton Research and Protection Council (ACRPC) and is considered a minor crop product in terms of crop value compared to corn, for example. AF-36 is produced at the ACRPC offices in Phoenix, Arizona. The ACRPC charges US$5.00 an acre for treatment of cotton fields in Arizona. AF-36 would not be suitable for use in fields outside Arizona, according to Peter Cotty. Thus this method is highly specific/location sensitive.

Despite being a natural product isolated from soil, it still took several years for AF-36 to be approved (Jones 2003). According to Larry Antilla of the ACRPC, the ACRPC's greatest problem today is complacency by their client growers, some of whom must be reminded that AF-36 treatment is a form of continuing insurance against aflatoxin contamination (pers. comm., 2006).

Symbiotic prevention of tooth decay

Oragenics (http://www.oragenics.com) has developed an innovative form of tooth decay prevention. Their website contains the following description:

> Most human tooth decay is caused by a bacterium called *Streptococcus mutans* that sits on the tooth surface and converts sugar in our diet to lactic acid. The lactic acid is excreted by the bacteria and causes tooth decay by dissolving the mineral that comprises our enamel and dentin. Our scientists have isolated a strain of *S. mutans* that produces a small amount of an antibiotic that is capable of killing all other strains of this species. Through

> recombinant DNA technology, we succeeded in eliminating the gene in this strain that is responsible for producing lactic acid. Consequently, it does not cause significant tooth decay.

The website goes on to describe 30 years of trials protecting rats from tooth decay using the recombinant *S. mutans* in "replacement therapy." In the presence of processed sugar that triggered tooth decay in control animals, the treated animals were protected. The Food and Drug Administration (FDA) of the USA approved human trials on 30 November 2004, but the conditions for the trials were so severe that they were never carried out: the FDA would only approve human trials if the company used subjects less than 55 years old, in perfect health and missing all of their natural teeth.

The possibility of locating another country that might have a friendlier regulatory climate was addressed, but it seems that many countries rely on the regulatory apparatus in the USA for guidelines for their own registration processes. Therefore, it has not been possible from 30 November 2004 to today to launch trials, and this innovative new technology continues to be stymied by regulatory fiat.

Symbiotic control of Pierce's disease

Pierce's disease (PD) of grapevines is caused by a xylem-limited bacterial pathogen strain from the complex *Xylella fastidiosa* (XF). The pathogen is transmitted between host plants by xylem-feeding insects, especially leafhopper insects called sharpshooters. One particular vector insect, *Homalodisca vitripennis*, the glassy-winged sharpshooter (GWSS; Takiya *et al*. 2006), is thought to be responsible for an epidemic of PD that occurred in the Temecula, CA, USA grape and wine growing area in 1997 (Blua & Morgan 2003). The GWSS population in California resembles most closely the one in Texas, which is within the evolutionary area of origin of both that extends from Florida to northeastern Mexico (Smith 2005). Although citrus is widely considered to be a preferred overwinter host for GWSS, citrus appears not to harbor the strain of *Xylella* that causes PD (Costa *et al*. 2004).

Despite dozens of research projects aimed at PD, the exact cause of symptoms in grapevines and the exact nature of transmission by the GWSS remain unclear.

The genome sequence of the XF strain causing Citrus Variegated Chlorosis (CVC-XF) in Brazil and the strain causing PD (PD-XF) in North America and Mexico have been reported (Simpson *et al*. 2000; Van Sluys *et al*. 2003). However, we do not know the genetic traits or genome sequences that cause a particular strain of XF to be pathogenic to a specific variety of plant.

We do know that wild muscadine grapevines in the primordial origin of PD, the southwestern USA and adjacent areas of Mexico, are resistant to PD despite the presence of PD-XF and the presence of insect vectors capable of transmitting the pathogen. Indeed, it is difficult to grow European grapevines, *Vitis vinifera*, in most of this endemic area; the hill country of Texas is a notable exception. We also know that certain grapevines are more likely to show symptoms of PD-XF than others. Indeed, there may be more than one strain of PD-XF causing PD.

Pierce's disease can be induced in susceptible grapevines by needle inoculation with a specific strain of XF. The CVC-XF strain was reported to cause PD when needle inoculated into grapevines. But in nature, the normal means of transmission of the pathogen is by sharpshooters and a few other plant sucking insects. We suspect the transmission is physical because of the short time between acquisition from an infected plant and successful transmission to another host plant. Nor does prolonged feeding appear to be necessary for transmission of the pathogen (Bextine *et al*. 2004). Feeding attempts by the GWSS *Homalodisca vitripennis* result in PD-XF being found in saliva at probe marks in plastic containers (Ramirez *et al*., unpubl. data, 2007).

Labavitch *et al*. (2005) reported that "polygalacturonase (PG), an enzyme produced by *Xylella fastidiosa* (PD-XF) actually contributes to development of Pierce's disease symptoms in inoculated grapevines." And that "the size of vessel pores increases with XF infection, presumably allowing bacterial cells to pass from vessel to vessel, and thus to spread to new tissues to eventually kill the plant. When XF-derived PG and endoglucanase are together artificially introduced into grapevine stems, the same increase in porosity occurred."

Labavitch *et al*. (2005) also reported functional elimination of the ability of PD-XF to synthesize PG by inserting an interrupting sequence in the coding region of the PG gene of PD-XF. The resulting recombinant PD-XF* strain lost the ability to produce PD symptoms both *in vitro* and in grapevines. A study by Agüero *et al*. (2005) showed that transgenic insertion of a pear fruit PG-inhibiting protein into grapevines led to decreased symptoms of PD in grapevines inoculated with PD-XF.

Agüero *et al*. (2005) also reported that PD-XF must pass through pit membranes of the xylem vessels. A point of contention was whether the pits were already open or not. If they were not open, they would require digestion, as suggested by Labavitch *et al*. (2005). If they were already open, the pathogen would spread in the grapevine without obvious impediment. Shackel and Labavitch (2005) concluded that the symptoms of PD were due to the plant response to PD-XF presence, not due to physical clogging of the xylem vessels. Elsewhere, Andersen and Leite (2005) claimed that pathogenicity was largely due to occlusion of xylem vessels by aggregates of XF cells and biofilms. They showed that aggregation of XF could be increased by certain concentrations of calcium and

magnesium chlorides. They suggested that the fluid environment altered the properties of the cell surface of XF affecting aggregation and thereby physical clogging of the xylem vessels.

Gabriel (2005) reported that genomic sequences of the Temecula strain of PD-XF contained no type III secretion sequences, but two type I secretion systems. Exchange mutagenesis that disrupted the secretion mechanism produced PD-XF* strains incapable of producing PD symptoms. Gabriel (2005) concluded that the pathogenic properties of PD-XF strains depended on type I secretions. This seemed to support the Labavitch view that secretions by PD-XF are responsible at least in part for PD symptoms. However, mutagenically altering the *tolC* gene in PD-XF reportedly had a fitness cost, so it is difficult to determine which factor contributed to the lack of symptoms in plants injected with *tolC*$^{-}$ PD-XF (the pathogenic *Xylella* lacking the *tolC* gene) – the effect on pathogenicity or the effect on general viability. Also, one would expect a secretion mechanism to export a number of products from bacteria, some of which might be transcription factors dictating responses in the plant that contribute to symptoms.

Lindow (2005; Newman *et al*. 2004) came to the apparent opposite conclusion: "Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of XF and that disruption of cell signaling is a promising means of controlling PD." He found PD-XF strains lacking the ability to signal cannot be transmitted by nor colonize sharpshooter insects. He studied a signaling molecule called DSF, an alpha, beta unsaturated fatty acid that coordinates gene expression.

A common theme for this lecture is that despite the enormous increase in data and information, we are humbled by how little we understand and how much there is to learn. This is the case with the cause of PD in grapevines.

The more detailed the genetic analyses, the less certain the species boundary. It seems that a given species is an organism that is one component of a metaorganism that is understood only as the sum of its components. So the "new biology" is no longer about single organisms, but about each organism as a symbiotic mixture or metaorganism.

Migratory locusts

Migratory locusts, including the desert locust *Schistocerca gregaria*, are a problem of great economic significance, but the most difficult aspect of that pest problem is the complacency that seems to occur in between swarm and migration events. In addition, the main countries afflicted have few economic resources to mount major efforts to fight off invasions and have to rely on others to help, such as the Food and Agriculture Organization (FAO) of the United Nations and a variety of organizations from individual countries. Malika Bounfour, who now works for the Ministry of Agriculture in Morocco, recently initiated an inquiry into new methods for dealing with the desert locust, which invades Morocco on a regular basis as migrating swarms from neighboring countries.

I have invited a group of people to address the possible development of new tools to control the desert locust that are based on biotechnology of the kinds described above. There are promising new directions to consider. Barbara Glenn, Managing Director, Animal Biotechnology of Biotechnology Industry Organization (BIO), Washington, DC, recently was quoted about the locust problem, "Frankly these are just the types of problems for which a biotech solution is so suitable and so compelling". A page of the website http://biopesticide.ucr.edu is reserved for developing a consensus. We invite any interested individuals or governments to participate.

Conclusions

While molecular biology and genomics have provided unprecedented detailed descriptions of nucleotide sequences in a number of organisms, translation of this information into practical pest and disease controls has been slow. The technical barriers to developing new methods are matched by regulatory uncertainty. Still, for many pest and disease problems, biotechnology is the only approach offering possible new solutions.

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