Microbial Toxins in Insect/Nematode Biocontrol

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Invertebrate pests, such as insects and nematodes, not only cause or transmit human and livestock diseases but also impose serious crop losses by direct injury as well as vectoring pathogenic microbes. This is amply demonstrated by the successful and widespread use of *Bacillus thuringiensis* (*Bt*) to control mosquitos and many plant pests, the latter by the transgenic expression of *Bt*-insecticidal proteins in crop plants. Identifying as well as characterizing the molecular nature and regulation of the biocidal activity has led to the enormous success of *Bt* as a biocontrol agent, which serves as a great model for advancing nascent biocontrol agents into commercial products.

Keywords: pore-forming toxins ; insect ion channel modulators ; innate immunity busters ; cyclic lipopeptide surfactants ; psychoactive compounds ; ribotoxins ; sterol homeostasis disruptors ; uncouplers

1. Introduction

Insects constitute the largest and most diverse group of animals (~2.5 million species) on Earth [1]. With the exception of a few beneficial insects such as pollinators, they form the costliest animal group to human society by spreading devastating infectious diseases in humans, livestock and crops, ravaging food stocks, damaging forests, destroying infrastructure and weakening the resilience of ecosystems [1]. The Nematoda (also called Nemathelminthes), with an estimated 500,000 species, is the second largest phylum in the animal kingdom [2]. Most species of nematodes are either innocuous or play beneficial ecological and agronomic roles, by nutrient recycling and controlling insects and other harmful nematodes [3]. At the same time, some nematodes cause serious diseases in plants, humans and other animals.

Biocontrol is the means of controlling pests and pathogens through the use of other organisms, which can be natural enemies, such as predators, parasitoids, pathogens and competitors. It is an environmentally safe, low-cost and effective approach and occurs in natural communities. Much before the introduction of chemical pesticides, biocontrol has been in practice to control agricultural pests, with the first recorded report in 304 AD from China [4].

In spite of clear advantages in terms of environment and food safety, microbials have not replaced chemical pesticides or become a major component of integrated pest management in intensive agriculture or the management of human and livestock health, the only exception being the use of *Bacillus thuringiensis* in mosquito control. Reasons for the limited success of biocontrol agents (BCAs) in pest control may include poor adaptation of a BCA to a new host, extensive non-target effects of the biocontrol microbe [5], lack of methods to mass produce fastidious BCAs in synthetic media, e.g., *Paenibacillus popilliae* [6], and environmental risks associated with the BCA, e.g., vancomycin resistance in *P. papillae* [7]. These concerns, exacerbated by the lack of efforts to clearly validate the potential environmental risks, have slowed down the use of microbials in pest control [8]. Many of these constraints can be lessened when the identity of a BCA's biocidal activity and its mode of action are known, as evident by the enormous success of *Bacillus thuringiensis* crops (*Bt*-crops: crops that are genetically modified to express *crystal* (Cry) proteins of *B. thuringiensis*) [9]; (**Figure 1**).

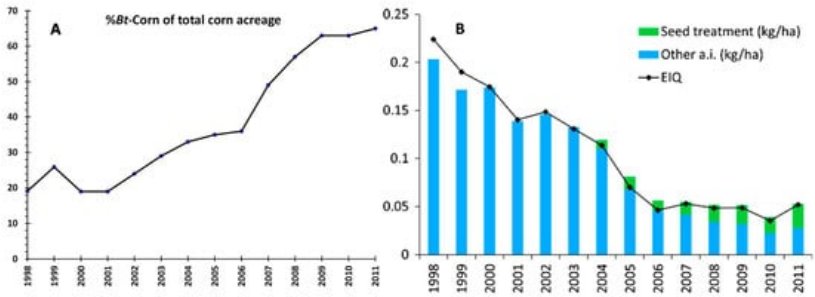

Figure 1. Insect resistant *Bt*-corn decreased pesticide use in US. (**A**) Percentage of *Bt*-corn acreage out of total corn planted between 1998 and 2011 (data extracted from [10]). (**B**) Insecticide use in maize (kg/ha and environmental impact quotient (EIQ) weights) during the same period as in A. (Figure 1B is reproduced from [11]).

Detailed analysis of microbial toxins not only helps in strain improvement of candidate BCAs but also would enable the assessment of their non-target effects and modify target specificity, mass produce the toxins in heterologous systems, incorporate insecticidal activity in crops, develop synthetic versions with enhanced toxicity and specificity, ensure consistency of insecticidal action when needed and develop formulations for potential deployment as tank mixes.

2. Pore-Forming Toxins

Pore-forming toxins (PFTs) are the largest class of proteinaceous bacterial toxins and important virulence factors, such as colicins of *E. coli* and anthacin of anthrax. All PFTs are synthesized as water-soluble proteins but subsequently become membrane bound. They recognize host cells based on specific cell surface receptors, which can be proteins, lipids or sugars. The binding allows a rapid increase in the local concentration of a PFT and its oligomerization, which is followed by insertion and pore formation in the host cell membrane. The changes in the permeability of the membrane due to pore formation depends on the toxin, ranging from the loss of small ions, e.g., K^+ and Ca^{2+}, to macromolecules such as proteins [12].

B. thuringiensis (*Bt*, hereafter) was first isolated in Japan in 1902 from dead silkworm (*Bombyx mori*) larvae and soon recognized as an entomopathogenic bacterium. *Bt* was one of the first prokaryotic BCAs used as a commercial insecticide in France in 1938 [13]. The insect- and nematode-specific pathogenicity of Cry toxins is due to their oral toxicity, and these toxins are active against a wide range of insects from Lepidoptera, Coleoptera, Hymenoptera and Diptera, as well as nematodes [14][15].

Genetic engineering technology enabled the introduction of genes encoding Cry and other *Bt*-insecticidal proteins into major crops. The first *Bt*-crop (maize) was approved for cultivation in 1995 in the US and its success has allowed a widespread cultivation of *Bt*-maize and other crops in the US and 18 other countries across the globe. However, there are many insect pests that show no or limited susceptibility to Cry toxins, and those susceptible develop resistance. Thus, there is a need to explore for novel insecticidal proteins as well as engineer variants of existing ones by in vitro protein evolution for enhanced toxicity, specificity and target range.

PFTs are not only made by bacteria but also are increasingly being discovered in eukaryotes [16], including as components of vertebrate immune systems [17][18]. The aegerolysins comprise over 350 small (~15–20 kDa), β-structured proteins found in several bacteria and eukaryotes. They are known to interact with specific membrane lipids and lipid domains and have recently shown to be a new class of PFTs.

3. Insect Ion Channel Modulators

Ion channel modulators have clear advantages as pesticidal targets in that they act quickly and allow the rapid reduction of insect pressure. Five ion channels within the insect nervous system have been the primary targets for the development of small molecule insecticides and also serve as effective targets for biopesticides. These are the γ-aminobutyric acid (GABA) receptor, the glutamate-gated chloride channel, the nicotinic acetylcholine receptor or nAChR, the voltage-gated sodium channel and the ryanodine receptor [19].

Nodulisporic acid A, a natural insecticidal indole terpene isolated from an endophytic fungus, *Nodulisporium* sp., also inhibits only invertebrate-specific glutamate-gated chloride (Glu-Cl) channels [20]. Based on its safety, Merck & Co. introduced synthetic versions of the compound as a potent oral formulation to control fleas and ticks in dogs and cats [21]. Ryanodine receptors (RyRs) belong to a group of ligand-gated calcium channels initially reported from vertebrates. They are located on the endoplasmic reticulum of muscle cells and neurons and play a critical role in muscle contraction. In contrast to mammals, which have three types of RyRs, insects have only a single RyR, which is a major target for modern insecticides. Ryanodine, a plant alkaloid and an important ligand of RyR, has served as a natural botanical insecticide. Attempts to generate synthetic commercial analogs of ryanodine have, so far, not succeeded. Despite the popularity of diamide insecticides due to their specificity, several agricultural pests have become resistant to the chemical insecticides due to mutations in a transmembrane region of their RyRs [22], and there is a need for alternative RyR agonists. Cyclodepsipeptides are a large family of peptide-related natural products with an α-hydroxy acid and 5–10 amino acids linked by amide and ester bonds [23]. Verticilide, a cyclooctadepsipeptide produced by *Verticillium* sp. FKI-1033, has been shown to bind selectively to the insect ryanodine receptor in the low micromolar range [24].

4. Innate Immunity Busters

Insects, like all invertebrates, express both innate and humoral immunity to infection. Biological control strategies that are targeted to these pathways have a great potential for success.

Bacteria belonging to 24 species of *Xenorhabdus* and five of *Photorhabdus* are known worldwide for their entomopathogenic potential. Some species (e.g., *P. luminescens* and *X. nematophila*) have mutualistic associations with nematodes and share their entomophagous lifestyle. The soil-dwelling entomopathogenic nematode larvae find and penetrate the insect larvae through natural openings and release symbiotic bacteria into the insect hemocoel. The bacteria replicate in the insect body and release immuno-suppressive toxins. At least seven secondary metabolites that inhibit PLA2, namely, benzylideneacetone (BZA), proline-tyrosine, acetylated phenylalanine-glycine-valine, indole, oxindole, cis-cyclo-PY and p-hydroxyphenyl propionic acid, have been reported. They also show significant inhibitory activities against other immune responses, such as phenoloxidase activity (PO) and hemocytic nodulation, with BZA being the most effective [25]. An isocyanide-containing compound rhabducin produced by *Xenorhabdus* can also inhibit phenoloxidase and thereby melanization [26]. In addition, phurealipids (urea compounds) made by these bacteria can prevent the expression of antimicrobial peptide genes in the insect hosts, a part of the humoral component of immunity [27].

5. Cyclic Lipopeptide Surfactants

Cyclic lipopeptides (CLPs) are amphiphilic molecules composed of a cyclic oligopeptide lactone ring coupled to a fatty-acid tail. Their structural diversity and cyclic configuration confer them with broad-spectrum and environmentally stable antibiosis activity against bacteria, fungi, insects, protozoa and even human tumor cell lines [28][29]. CLPs are produced mostly by *Pseudomonas*, *Bacillus* and *Streptomyces* spp. CLPs come in three families, namely surfactin, iturin and fengycin, and a single strain can make one or more of them contribute to varying biocidal activities. *Pseudomonad* spp. (e.g., *P. chlororaphis*, *P. fluorescens*, *P. protegens*, *P. putida*, *P. mosselli* and *P. entomophila*) are particularly pathogenic toward insects and nematodes.

6. Psychoactive Compounds

Many microbial parasites manipulate host insect behavior for the enhanced dispersal of their inoculum and thereby spread disease. A few of them, such as baculoviruses, hijack host genes involved in insect physiology [30], whereas others make their own [31]. Many entomopathogenic fungi cause “summit disease” (SD) behavior, an extended phenotype where parasitized insects ascend and affix to elevated substrates prior to death. For example, the fungal pathogen *Entomophthora muscae* infects wild *Drosophila* and manipulates host behavior [56]. This facilitates a wider dissemination of fungal spores from the mummified insect carcasses. It is speculated that *E. muscae* may produce and secrete eicosanoid-like compounds to induce behavioral changes in the dying host [32]. More details are available about the bizarre behavior in cicadas induced by the pathogenic fungi *Massospora* and *Strongwellsea*. *M. cicadina*, *M. platypediae*, *M. levispora*, *S. tigrinae* and *S. acerosa* keep their insect hosts alive while sporulating, which enhances dispersal via sexual transmission.

More research is needed to validate the findings from -omics analyses to unequivocally implicate any of these putative chemosignaling compounds in insect behavioral manipulation. Nevertheless, this concept has been successfully exploited in the management of some pests, e.g., codling moth (*Cydia pomonella*, a key insect pest of apple), using host endogenous molecules, especially pheromones [33].

7. Ribotoxins

Ribosome-inhibiting proteins (RIPs) or ribotoxins are produced by *Aspergillus* and *Penicillium* spp. and also by some entomopathogenic fungi, e.g., *Hirsutella thompsonii* or *Metarhizium anisopliae* [34]. RNase T1 is the best-known representative of this group of cytotoxins. Ribotoxins can enter the cell by crossing lipid membranes without the need for a protein receptor via their ability to interact with acid phospholipid-containing membranes. Once inside the cell, they cleave a single phosphodiester bond located within the sarcin–ricin loop, a universally conserved sequence of the large rRNA gene. This leads to the inactivation of ribosomes, the inhibition of protein biosynthesis, followed by cellular death by apoptosis [35]. *Hirsutella thompsonii* infects different types of insects as well as mites and nematodes, particularly causing spectacular natural epizootics among mite populations [36].

8. Sterol Homeostasis Disruptors

Most insects are sterol auxotrophs and depend on dietary sterols for their structural and metabolic needs. Therefore, cholesterol uptake, transport and metabolism can be potent targets for vector and pest control strategies [37]. A secreted cholesterol oxidase from *Streptomyces* shows strong insecticidal activity against boll weevil larvae [38]. Pyripyropenes from the fermentation broth of Aspergillus 317 fumigatus (an opportunistic human pathogen that causes invasive

pulmonary 318 aspergillosis) are also strong inhibitors of acyl-CoA:cholesterol acyltransferase. One of the derivatives showed far superior activity to that of the natural compound on aphids and was commercialized as afidopyropen (**Figure 2**) [70], demonstrating the power of synthetic chemistry tools in product discovery.

Figure 2. A derivative of natural pyripyropene shows superior aphicidal activity. Of >40 derivatives tested, the derivative with cyclopropanecarbonyloxy groups at the C-1 and C-11 positions and a hydroxyl group at the C-7 position showed the highest insecticidal activity against aphids [39].

9. Uncouplers and Electron Transport Inhibitors

Natural compound inhibitors of the mitochondrial electron transport chain and uncouplers of oxidative phosphorylation are promising candidate insecticides if they also prove to be highly selective and environmentally safe. The well-known example is the isoflavone rotenone, a complex I inhibitor from plants. It is a broad-spectrum insecticide and piscicide, due to its mild toxicity to humans and other mammals [40]. A few microbial toxins with insecticidal properties show this mode of action. A common soil hyphomycete, *Purpureocillium lilacinum* (syn. *Paecilomyces lilacinus*), is used as a nematicide. However, it can cause rare opportunistic infections in humans and also has significant resistance to conventional antifungals [41]. It secretes a nematicidal toxin called paecilotoxin. The toxin belongs to the leucinostatins, a class of neutral straight peptides containing an unsaturated fatty acid at the N-terminus, and has strong uncoupling activity via its inhibition of the membrane-bound component of ATP synthases [42]. Overproduction of the toxin in fermentation may allow large-scale field testing for its pesticidal efficacy and environmental safety [43].

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