Bulletin of Insectology ***66*** *(2): 181-186, 2013*
ISSN 1721-8861

Emerging entomopathogenic bacteria for insect pest management

Luca Ruiu, Alberto Satta, Ignazio Floris
Dipartimento di Agraria, Sezione Patologia vegetale ed Entomologia, Università degli Studi di Sassari, Italy

Abstract

The scientific community working in the field of insect pathology is experiencing an increasing academic and industrial interest in the discovery and development of new bioinsecticides as environmentally friendly pest control tools to be integrated, in combination or rotation, with chemicals in pest management programmes. In this scientific context, market data report a 15% annual growth of the biopesticide segment. This trend is in line with the requirements of new regulations on Integrated Pest Management. After few decades of research on microbial pest management dominated by *Bacillus thuringiensis* (*Bt*), novel bacterial species are being discovered and developed into new products. With the aim to give a timely picture of the cutting-edge advancements in this renewed research field, different representative cases are reported, especially including *Brevibacillus laterosporus*, *Chromobacterium subtsugae* and *Yersinia entomophaga*.

Key words: microbial control, insect pests, entomopathogenic bacteria, *Bt*, bioinsecticides.

Overview

Pest management approach, methods and discipline have experienced over time developments and advancements to minimize environmental impact.

The global pesticide market is presently growing at a rate of 3.6% per year and is valued around $ 47 billion (BCC Research, 2010). This trend is in relation to the need of protecting the environment, farmlands and the agriculture crops (Oerke and Dehene, 2004) feeding a human population expanding at a rate around 1.15% per year (United Nations, 2011).

In this context, environmentally sustainable improvements in technology, agricultural techniques, and pest management are vital to allow farmers to expand crop production and animal farming on the limited land available.

Since after the Second World War, the control of pests in agriculture and in animal husbandry has mostly relied on the intensive application of synthetic insecticides. Despite being a successful strategy, the concerns for the environment and human health have stimulated a continuous research and development of more environmentally responsible alternatives reducing the risks connected with chemicals. Among these, the insect resistance development, the destruction of non-target *entomofauna* including natural predators, parasitoids and pollinators, as well as the contamination of water and food. For these reasons the search for environmentally friendly control systems and their deployment in Integrated Pest Management strategies has been a main objective for both academia and industry during the last decades. As a result of these efforts, an increasing interest in the use of successful biological control strategies was recorded, with special regard to the formulation of specific insecticides. This approach led to the progressive discovery and use of various entomopathogenic microbial species including bacteria, virus, protozoa, fungi, microsporidia, nematodes (Vega and Kaya, 2012). Most of these entomopathogens are normally able to persist in the environment, to multiply in the host and to spread to other susceptible hosts. They have developed different strategies to attack, to enter and to kill the host. Typically, insect mycopathogens enter through the cuticle whereas virus, bacteria and protozoan enter through the midgut. Nematodes exploit natural openings on the host body. The present work focuses mainly on entomopathogenic bacteria, leaving aside the other insect pathogenic microorganisms that are anyway of significant importance for the future development of new bioinsecticides.

On the other side, the purpose of the present paper is not to be an exhaustive review of all the novel bacterial species and strains that are being discovered and developed into products, but to give a timely picture of the cutting-edge advancements in this fast growing discipline. In the envisioned, post-*Bacillus thuringiensis* era, some representative cases regarding emerging entomopathogenic bacteria other than *Bacillus* are presented and discussed. These include the case of *Brevibacillus laterosporus* which is object of our research studies.

Development of entomopathogenic bacteria for pest management

Among the first significant experiences with microbial control methods are the studies with the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin at the end of the XIX century, whose bioinsecticidal action is related to its conidia germination and ife penetration inside insect body (Pekrul and Grula, 1979). Later on, different fungal species were involved in biological control experimentations producing variable results. Among these, species belonging to the genera *Aschersonia*, *Agerata*, *Verticillium*, *Sphaerostilbe*, *Podonectria*, *Myriangium*, *Hirsutella*, *Metarhizium* (Fawcett, 1944). The biological control paradigm changed some decades later, when the potential of entomopathogenic bacteria was discovered, especially associated to species belonging to the genus *Bacillus* (Glare and O'Callagan, 2000). Initially, the species *Paenibacillus* (former *Bacillus*) *popilliae* Dutky was introduced for the management of the Japanese Beetle *Popillia japonica* Newman (Steinhaus, 1975), but more concrete results were achieved with the discovery of new *Bacillus thuringiensis* (*Bt*) strains showing high toxicity against specific insects at competitive level compared to conventional insecticides in terms of efficacy and costs of production. The strain HD-1, belonging to subsp. *kurstaki* (De Barjac and Lemille, 1970), soon became the main commercial focus for the management of lepidopteran pests in agriculture and forestry. Beside it, today other strains are commercially available, such as SA-11, SA-12, PB 54, ABTS-351 and EG2348, all isolated from insects or soil, and expressing a range of different toxins mostly belonging to the Cry1 and Cry2 families. Subsequently, the discovery of a *Bt* strain belonging to the subsp. *israelensis* (*Bti*) was followed by its commercialization for the management of mosquitoes and simulids (Goldberg and Margalit, 1977). Then, a particularly active strain of the subsp. *tenebrionis* was discovered and employed against coleoptera (Krieg *et al.*, 1983).

Undoubtedly, *Bacillus thuringiensis* is the species on which most of the scientific community and industry efforts have been focused. Main feature of this bacterium is the production of parasporal bodies (crystals) containing specific insecticidal endotoxins (Cry proteins) acting by ingestions through a pore-forming mechanism of action detrimental for the insect gut epithelium (Pigott and Ellar, 2007).

With reference to a strain-specific mode of action, Cry toxins act only if binding to gut receptors varying among insect species. For these reasons, an increasing number of *cry* genes have been identified and sequenced, and more genes continue to be discovered as new bacterial isolates are collected worldwide (De Maagd *et al.*, 2003). Other protein toxins from *Bt* (i.e. Cyt, VIP) have been identified and their genes characterized. As a result of this continuous search, an official international database including toxins and genes from *Bt* and other etomopathogenic bacteria is continuously being updated (Crickmore *et al*., 2006).

Different studies to evaluate the effects of *Bt* toxins on both insect pests (Pérez-Guerrero *et al*., 2012) and on non-target species (Marchetti *et al*., 2012) are continuously being conducted.

Besides *Bt*, other *Bacillus* species have shown potential for insect pest management. This is the case of *B. sphaericus* that represents a heterogeneous group including spherical endospore producing bacteria (Alexander and Priest, 1990). Strains belonging to this species are toxic against mosquitoes as a result of the production of parasporal crystals located within the exosporium and closely associated with the endospore. These parasporal bodies contain potent binary protein toxins (Bin), including an equimolar ratio of the two homologous BinA and BinB, acting in a similar fashion as Cry proteins (Broadwell and Baumann, 1987; Charles *et al.*, 2000). During the vegetative phase, *B. sphaericus* is also capable to produce mosquitocidal toxins known as Mtx proteins.

Homology among toxins from *B. sphaericus* and toxins from *Bt* or other entomopathogenic bacteria has been shown, demonstrating their phylogenetic relationships and revealing a probable common co-evolution (de Maagd *et al*., 2003).

There are many entomopathogenic bacteria that have shown potential against diverse insect pests, but have not achieved the same commercial success as *Bt*. Among these, the previously cited *P. popilliae* with *P. lentimorbus*, the causal agents of milky disease in phytophagous scarab larvae (Zhang *et al.*, 1997), and *Serratia entomophila* (Enterobacteriaceae) containing a specific plasmid (pADAP) encoding genes implied in the pathogenicity against the grass grub, *Costelytra zealandica* (White) (Coleoptera Scarabaeidae) (Jackson *et al*., 1992).

The *Paenibacillus* genus, includes also *P. larvae* (White), the etiological agent of one of the main bacterial honey bee pathology, the American Foulbrood, that is object of continuous research aimed at studying the host-pathogen relationship and developing new eco-sustainable management methods (Gende *et al.*, 2010; 2011).

Entomopathogenic bacteria other than *Bacillus*

Among the wide variety of bacteria associated to insects, there are different examples of entomopathogenic species other than *Bacillus* that have been studied at different levels.

These include, for instance, *Clostridium bifermentans* serovar *malaysia* active against mosquitoes and blackflies (Nicolas *et al.*, 1990). In this case, the mosquitocidal activity has been associated to the production of a protein with homology to *Bt* delta endotoxins (Cbm71). To prove the role of this protein, the encoding gene was cloned and induced to be expressed by transformed *Bt,* which exhibited toxicity against mosquitoes (Barloy *et al.*, 1996).

Another group of entomopathogenic bacteria with high interest is represented by the endosymbionts of insecticidal nematodes, especially the members of the genera *Xenorhabdus* and *Photorhabdus*. The first is associated to nematodes in the genus *Steinernema*, while the second colonize the intestines of *Heterorhabditis* species. Normally, after nematodes invade susceptible insect hosts, symbiotic bacteria are released in the hemocoel where they produce various virulence factors contributing to impair insect immune system and to kill the host. Comparative genomic studies on different *Xenorhabdus* and *Photorhabdus* species (*X. nematophila*, *X. bovienii*, *P. luminescens*, *P. asymbiotica)* highlighted the presence of numerous genes implied in the insecticidal action (Chaston *et al*., 2011).

Significant is also the case of *Pseudomonas entomophila*, an ubiquitous bacterium showing insecticidal properties against insects in different orders and which has the capacity to trigger a systemic immune response in *Drosophila melanogaster* Meigen after ingestion (Vodovar *et al*., 2005). The whole genome of this bacterium has been sequenced and different insecticidal toxin complexes have been identified (Vodovar *et al*., 2006).

Due to specific biological and technical aspects, such as the specific mode of action, commercially available strains, including *Bt*, have their restrictions in terms of performance in field conditions. Therefore, the continuous screening for new bacterial isolates is leading to the discovery of novel environmentally safe microbial insecticides, which can create new opportunities for the management of certain insect pests. Three representative cases regarding emerging bacterial species are presented.

Brevibacillus laterosporus

Brevibacillus laterosporus Laubach is a spore former belonging to the *B. brevis* phylogenetic cluster and is morphologically characterized by the production of a canoe-shaped lamellar body attached to one side of the spore also after lysis of the sporangium (Shida *et al*., 1996) (figure 1). It's a natural inhabitant of water (Laubach, 1916), soil (Oliveira *et al*., 2004; Ruiu *et al*., 2006), and has also been isolated from insects (McCray, 1917). It's biopesticidal potential has been reported against insects in different orders, such as Coleoptera (Boets *et al*., 2004), Lepidoptera (Oliveira *et al*., 2004), mosquitoes and black flies (Favret and Yousten, 1985; Rivers *et al*., 1991), house flies (Ruiu *et al*., 2006), against nematodes (Bone *et al*. 1991; Singer, 1996) and against phytopathogenic fungi (Saikia *et al*., 2011). This microrganism, can occasionally be found in honey bee larvae affected by European foulbrood (EFB), although it is considered to be just one of the secondary invaders encountered in the disease (Bailey, 1963; Gilliam and Valentine, 1976, Alippi, 1991).

Different insecticidal toxins produced by diverse *B. laterosporus* strains have been identified and characterized against various targets. For instance, Bone *et al*. (1991) isolated from the spore a heat stable low molecular weight protein inhibiting nematode juvenile development. Boets *et al*. (2004) highlighted the insecticidal role of secreted binary toxins (ISP1A and ISP2A) displaying homology with *Bt* Cry proteins acting against different coleopteran species (*Diabrotrica* spp., *Leptinotarsa* spp. and *Anthonomus* spp.).

More recently, certain mosquitocidal *B. laterosporus* strains were discovered to produce insecticidal crystals similar to those typical of *Bt*. Proteins contained in these parasporal bodies were isolated and correlated to the insecticidal action of these strains (Zubasheva *et al*., 2010). Enhancement of this larvicidal activity was obtained by bioincapsulation in Protozoa (Zubasheva *et al*., 2011). On the other side, the toxicity against flies is in relation to spores of strains lacking parasporal crystals (Ruiu *et al*., 2007a).

As a result of recent studies, the ultrastructural effects of this entomopathogenic species was shown against house fly larvae exposed to a *B. laterosporus* treated diet. The progressive symptomathology and midgut changes induced in intoxicated larvae are similar to those caused by *Bt*, thus suggesting a toxin mediated process involving the alteration of midgut epithelial membrane permeability leading to osmotic disorders, cell alteration and disruption (Ruiu *et al*., 2012).

Promising field applications with experimental formulations in dairy farms against flies have been carried out (Ruiu *et al*., 2008 and 2011). The safety of this bacterium towards non-target species has also been investigated (Ruiu *et al*., 2007b and c).

Chromobacterium subtsugae

Firstly isolated from forest soil in Maryland (USA), *Chromobacterium subtsugae* is a gram negative betaproteobacterium developing violet-pigmented colonies, due to the production of violecein, a tryptophan derivative synthesized by different bacterial species (Hoshino *et al*., 2011). It was indicated as a novel species, as a result of 16S rRNA gene sequencing and phenotypic characterization, which allowed to distinguish it from *C. violaceum*, a species that shares the ability to produce violacein (Durán and Menck, 2001).

Its insecticidal potential by ingestion was initially proved against diverse insect species in different orders (i.e. Coleoptera, Lepidoptera, Hemiptera). These included the Colorado potato beetle (*Leptinotarsa decemlineata* Say), the Western corn rootworm (*Diabrotica virgifera* Le Conte), the Southern corn rootworm (*Diabrotica undecimpunctata* Mannerheim), the small hive beetle (*Aethina tumida* Murray), the diamondback moth (*Plutella xylostella* L.) the sweet potato whitefly (*Bemisia tabaci* Gennadium), the Southern green stink bug (*Nezara viridula* L.) (Martin *et al*., 2007a). In addition to mortality, significant sub-lethal effects, with special regard to feeding inhibition, were observed in most cases. Martin *et al*. (2007b) associated the insecticidal activity to either viable cells or cells killed by autoclaving, thus suggesting a mechanism of action mediated by heat-stable toxins. The stability of toxic factors was proved to be maintained also after freezing or pH changes (Martin *et al*., 2007c).

Successively, analytical studies on a crude extract combined with bioassays on the Beet Armyworm (*Spodoptera exigua* Hubner), confirmed the production of bioactive compounds during *C. subtsugae* stationary growth phase (Koivunen *et al*., 2009).

The mode of action of this new entomopathogenic bacterium is complex and different are the metabolites that have recently been associated to the insecticidal effects (Asolkar *et al*., 2012).

C. subtsugae strain PRAA4-1T has now been developed into a commercially available product (Grandevo, Marrone Bio Innovations Inc.) for the management of a broad spectrum of chewing and sucking insect species.

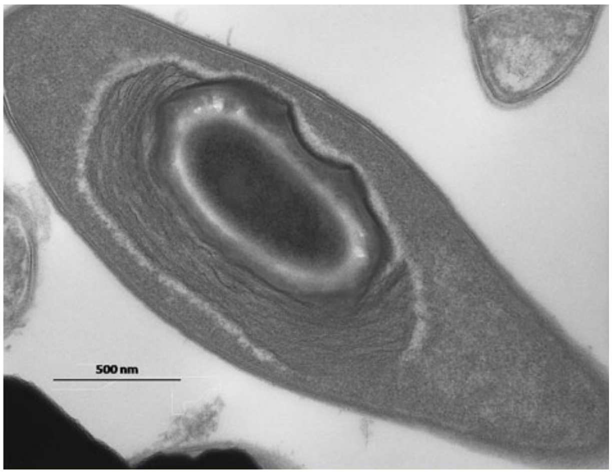

Figure1. *Brevibacillus laterosporus* sporangium.

Yersinia entomophaga

This recently discovered species was isolated from diseased larvae of the New Zealand grass grub, *Costelytra zealandica* White (Coleoptera Scarabaeidae) (Hurst *et al*., 2011a). It is a non-spore-forming bacterium that secretes a multisubunit toxin complex (Yen-Tc) showing homology with toxin complexes produced by *Photorabdus* spp. Proteins in the complex include three protein families termed A (YenA1, YenA2), B (YenB) and C (YenC1, YenC2) and two chitinases (Chi1 and Chi2) with high endochitinase activity (Hurst *et al*., 2011b). The structure of chitinases has been analyzed and different are the hypothesis formulated to explain their role in the toxin complexes. For instance, they may assist peritrophic membrane degradation in the gut, opening the way to ABC toxins, or they may play a role in the post-mortem degradation of insects, or they may anchor the toxin complex to the peritrophic membrane, avoiding it to be washed through the gut (Busby *et al*., 2012).

Toxin complex (Tc) proteins are a class of bacterial proteins exhibiting insecticidal activity. They were first discovered in *Photorhabdus luminescens*, an entomopathogenic nematode symbiont (Bowen *et al*., 1998), then Tc-like proteins were identified on other entomopathogenic bacteria such as *Serratia entomophila* (Hurst *et al*., 2000) and the nematode symbiont *Xenorhabdus nematophila* (Morgan *et al*., 2001). In the case of *Y. entomophaga*, 3D structure studies of the toxin complex showed that subunits YenA1 and YenA2 form the basis of a five fold symmetric assembly, while subunits B and C form a surface accessible region and are the main toxicity determinants. The chitinases decorate the surface of the TcA scaffold (Landsberg *et al*., 2011). These toxins have a broad insecticidal range of activity including Coleoptera and Lepidoptera. Histopathological studies on the effects caused by the ingestion of Yen-Tc revealed the progressive disorganization and deterioration of the midgut epithelium of *C. zealandica*. These effects are reminiscent of those previously reported for other Tc producing bacteria, like *P. luminescens* and *X. nematophila* (Marshall *et al*., 2012).

Recently, the insecticidal activity of formulations containing *Y. entomophaga* against the pasture pest porina (*Wiseana* spp. larvae) has been reported highlighting its persistence and survival in the field, thus suggesting its use as a microbial alternative for the management of porina (Ferguson *et al*., 2012).

Future directions and research needs

The recent achievements of the scientific community working in the area of insect pathology are contributing to increase the effort directed toward the discovery of new bacterial-based insecticides.

As recently reported by BCC Research (2010), the whole biopesticide market, including both microbial products and natural biochemicals, is estimated to grow at a 15.6% compound annual growth rate (CAGR) and in line with a recent report of Global Industry Analysts (2012), it is forecast to reach $ 2.8 billion by the year 2015.

In this scenario the interest in bioinsecticides is significantly growing, also as a result of the withdrawal of many synthetic pesticides and the high cost for the development of new ones, the new regulations reducing the maximum residue levels (MRLs) for synthetic pesticides and favouring the registration of low risk substances for pest control (i.e. EC Regulation No. 1107/2009). In addition, the implementation of Integrated Pest Management becomes compulsory by 2014 (EC Directive 2009/128).

Despite the success of many available products, the use of microbial based biological control is still relegated to niche contexts, in relation to the previously described highly specific mode of action and the narrow efficacy spectrum. Biopesticides use in combination or rotation with synthetic pesticides is likely to be enhanced in the near future, but more research is needed to come up with innovative solutions that can really meet farmers and regulator needs in terms of effectiveness and environmental sustainability. At the present state of the art, biopesticides have not yet achieved their potential due to the lack of truly transformational associated technologies that may enhance their effectiveness (Glare *et al*., 2012).

Significant is that the most original findings are the results of screening programmes involving the isolation of new microorganisms from the natural environment. This method is permitting to discover and name new entomopathogenic bacterial species, such as the reported *C. subtsugae* and *Y. entomophaga*. Through this approach, leveraging natural bacterial biodiversity, forthcoming original discoveries are expected.

On the other side, the modern "omics" technologies, including whole genomic sequencing, allow to better access different biological aspects and clarify the entomopathogen-host interaction up to the molecular level.

Do new research achievements in the field announce a post-*Bt* era, with a new generation of broad spectrum entomopathogens?

Acknowledgements

This study was financially supported by Sardegna Ricerche (Bioecopest Project: Incentivo StartUp Polaris 2010) and by the Italian Ministry of Education, University and Research (PRIN 2008 Project: Discovery and evaluation of new microbial and vegetable biopesticides for the natural insect pests control).

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Authors' addresses: Luca Ruiu (corresponding author, lucaruiu@uniss.it), Alberto Satta, Ignazio Floris, Dipartimento di Agraria, University of Sassari, Sezione di Patologia Vegetale ed Entomologia, via E. de Nicola, 07100 Sassari, Italy.

Received January 31, 2013. Accepted May 16, 2013.