BACTERIOLOGICAL CONTROL OF MOSQUITOES AND BLACKFLIES: PRESENT ASPECTS AND PERSPECTIVE OF RESEARCH

L. NICOLAS[1]

ABSTRACT - Biological control of mosquitoes and blackflies, which transmit severe diseases, rely mainly on *Bacillus thuringiensis israelensis (Bti)* and *B. sphaericus*. Their lack of toxicity to non-target organisms and to the environment has favored their development in vector control programs. *Bti* is now widely used in blackfly control as well as against most of the mosquito species. The host range of *B. sphaericus* is restricted to *Culex* sp. and to lesser extent to *Anopheles* sp. and some *Aedes* species. However, to the contrary of *Bti*, *B. sphaericus* toxicity persists in polluted water, therefore this bacterium is increasingly applied against the urban mosquitoes *Culex* sp. Recent advances in the knowledge of the bacterial toxins and in genetic engineering have opened the field of designing new formulations, modifying the host range of *Bti* or *B. sphaericus* and introducing toxin genes into microorganisms which serve as food for the larvae, e.g. cyanobacteria. Research undertaken in our laboratory on *Clostridium bifermentans* serovar *malaysia*, an anaerobic strain which has been recently demonstrated to be highly toxic to mosquitoes and blackflies, will be also discussed. This is the first time that an anaerobe toxic to Diptera is isolated and this allows to find new toxins, different of the known toxins of *B. sphaericus* or *B. thuringiensis*.

Index terms: Larvicides, biological control, *Bacillus thuringiensis*, *Bacillus sphaericus*, *Clostridium bifermentans*, mosquito control, blackfly control, increased host range, persistence, bioinsecticide formulation, biological insecticides.

CONTROLE BACTERIOLÓGICO DE MOSQUITOS E SIMULÍDEOS: ASPECTOS ATUAIS E PERSPECTIVAS DE PESQUISA

RESUMO - O controle biológico de mosquitos e borrachudos que transmitem graves doenças, é feito principalmente com *Bacillus thuringiensis israelensis (Bti)* e *Bacilus sphaericus*. A inexistência de toxicidade para outros organismos com os quais se relaciona no meio ambiente, favoreceu a sua utilização nos programas de controle de vetores. O *Bti* é utilizado não só no controle de simulídeos como também no combate a maioria das espécies de mosquitos. O espectro de ação do *B. sphaericus* é restrito ao gênero *Culex* com pouca toxicidade contra o gênero *Anopheles* e algumas espécies de *Aedes*. Contudo, ao contrário do *Bti*, a toxicidade *B. sphaericus* persiste em águas poluídas e por esta razão esta bactéria tem sido muito utilizada contra os mosquitos urbanos do gênero *Culex*. Recentes avanços no estudo das toxinas bacterianas e em engenharia genética abriram caminho para o desenvolvimento de novas formulações, modificando o espectro de ação de *Bti* ou *B. sphaericus* e introduzindo genes de toxinas em microorganismos que servem como alimento para os mosquitos como por exemplo cianobactéria. Será também discutida a pesquisa em andamento em nosso laboratório com *Clostridium bifermentans* serovar *malaysia*, uma linhagem anaeróbica que recentemente se descobriu ser extremamente tóxica para mosquitos e borrachudos. Esta é a primeira vez que um anaeróbico tóxico para dípteros é isolado e isto permite encontrar novas toxinas, diferentes das conhecidas de *B. sphaericus* e *B. thuringiensis*.

Termos para indexação: Larvicidas, controle biológico, *Bacillus thuringiensis*, *Bacillus sphaericus*, *Clostridium bifermentans*, controle de *Culex*, aumento persistente dos grupos, elaboração de bioinseticidas, inseticidas biológicos.

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[1] Institut Pasteur, Unité des Bactéries Entomopathogènes, 25 rue du Dr. Roux, 75724 Paris cedex 15. France.

INTRODUCTION

Mosquitoes (Diptera, Culicidae) and blackflies (Diptera, Simuliidae) are vectors of severe human diseases, such as arboviroses, malaria and filariasis, threatening more than three billion people in tropical and subtropical countries (WHO 1989). Vector control strategies have been mainly based since the 1940's on the use of a limited number of chemical insecticides, mainly organophosphorous and organochlorine compounds. Although the advantages of chemicals cannot be denied, extensive and often non rational application of increasing amounts of chemicals, have led to the development of resistance among target vector populations. In 1980, 51 Anophelinae species, 42 Culicinae species and 41 other medically important Arthropodes, were resistant to one or several chemical residual insecticides (WHO 1980). Since, numerous important Anophelinae species have developed a multiple resistance to organochlorines, organophosphorous or carbamates compounds (Miles 1985). In West Africa, *Simulium damnosum* and *Simulium soubrensi*, vectors of onchocerciasis, have become resistant to temephos (Guillet et al 1980) or to chlorphoxim (Kurtak et al 1982). The lack of specificity of these molecules have also contributed to the decline of certain natural enemies of vectors. For example, the non man-biting mosquitoes *Culex cinereus* and *Culex nebulosus*, competitors of *Culex quinquefasciatus* but more sensitive to chemical insecticides than this latter, have been eliminated from certain areas, therefore leaving new breeding sites at the disposal of *Culex quinquefasciatus* (Subra 1981). The development of insect resistance but also the reluctance of human populations to sprayings of non specific chemicals in houses, have favoured the integration of several techniques in vector-borne disease control, including development of drugs or vaccines, environment, sanitary education and use of biological control agents.

Among the biological .control agents of vectors so far tested in the field, success are obtained in some cases with larvivorous fishes but mainly with two entomopathogenic bacteria: *Bacillus thuringiensis* var. *israelensis* (or *Bti*) and *Bacillus sphaericus*.

These two bacteria are active as larvicides and therefore their application could be envisaged when a larvicidal control of a target vector can lead to reduction of the nuisance or of the transmission of disease.

Bti and *B. sphaericus* are aerobic spore forming bacteria synthetizing during the course of sporulation several endotoxins which aggregate in the cells in parasporal inclusion bodies called crystals. They can be grown in mass in fermentors. At the end of culture, the cells and crystals are harvested, and are spread, generally after formulation, on water where the larvae breed. After ingestion of the crystals by the target larvae, toxins are released from the crystals, due to the combined action of gut proteinases and alkaline pH, and cause disturbance of the gut epithelial cells, leading to the death of the larvae.

HOST RANGE AND FIELD APPLICATION OF *Bti* AND *B. SPHAERICUS*

In 1977, *Bti* was isolated in Israel and shown to be highly toxic to the main mosquito genera (Goldberg & Margalit 1977) and characterized as a new serotype (H14) of *Bacillus thuringiensis* (de Barjac 1978). The toxicity of *Bti* to the larvae of the blackfly *Simulium damsonsum* (Guillet & de Barjac 1979), vector of onchocerciasis, raised a great interest in the Onchocerciasis Control Program in West Africa. Initiated in 7 countries in 1974, this program was at this time only based on larvicidal control and rapidly was facing the problem of resistance to the initial insecticide, temephos (Guillet et al 1980). Cooperation was set up between WHO, OCP, public institutions and industries involved in

Bacillus thuringiensis production against crop pests. At present, due to the development of suitable formulations and to the innocuity of *Bti* to non-target organisms, more than 600.000 liters of *Bti* are used annually, in alternance with temephos, chlorphoxim, carbosulfan and permethrin (Lévêque 1990; Le Berre et al 1990).

Bti is toxic in the laboratory to mosquitoes belonging to genera *Culex, Anopheles, Aedes* and *Mansonia* (table 1). In the field, its toxicity is longer in clear water than in polluted water, due to aggregation of the crystals to suspended matter and to inactivation of the toxin by polluants. Several commercial formulations, such as wettable powders, liquid concentrates, granules, pellets and also floatable briquets are now available from the industry (Bactimos®, Vectobac®, Teknar® and Skeetal®) or have been prepared by mosquito control agencies. Asporogenic mutants of *Bti* have also been developed in particular for use in drinking water against *Aedes aegypti* larvae. Although, chemical larvicides still remain widely used in many countries, an increasing number of countries, greatly concerned by the protection of environment and the use of safe mosquito control measures, try to encourage the development of *Bti*. For example, mosquitoes in the Rhine Valley (West Germany) are almost exclusively controlled with *Bti* (Becker 1990).

Since the discovery of the first weakly mosquitocidal strain of *B. sphaericus* (Kellen K) in 1965, several strains of *B. sphaericus* highly toxic to mosquito larvae have been isolated. In general, the *B. sphaericus* are most active against mosquitoes of the genus *Culex* followed by *Anopheles sp.* and *Psorophora sp.* and are poorly active against *Aedes sp.* (Table 1). *B. sphaericus* strains 2362 and 1593 are also highly toxic to *Mansonia uniformis* (Yap et al 1988) while strain 2297 is not. *B. sphaericus* strains are neither toxic to blackfly larvae, nor to non-target organisms such as mammals and fishes. The limited host-range of *B. sphaericus*, has hampered until recently its interest in mosquito control. However, contrarily to *Bti*, *B. sphaericus* is very effective in polluted water, where its toxicity can persist for several weeks (Nicolas et al. 1987a). This is probably due to the fact that the crystal of *B. sphaericus* is linked to the spore by the exosporium and is released when the bacterial cells lyse as a spore/crystal complex, which confers a protection to the environment. On the opposite, the crystals of *Bti* are physically independant of the spore.

TABLE 1. Host-range of *Bti.* and *B. sphaericus.*

Genus	Culex	Anopheles	Aedes	Mansonia	Simulium
Most common biotope	Polluted water	Sunny clear water	Clear water	Clear water	. Rivers
Bti					
Laboratory	+++	++	+++		
Field	+	++	+++	++	+++
B. sphaericus					
Laboratory	+++	++	+/-		
Field	+++	+	+/-	++	-

Due to this asset, the use of *B. sphaericus* against *Culex quinquefasciatus,* which can breed in polluted water in urban areas, is increasing. A commercial formulation, Spherimos®, has been registered in 1989.

Local production of *Bti* and *B. sphaericus* is now carried out in several tropical countries, concerned by vector-borne diseases, such as Thailand, India, China, the Philippines or Brazil.

DRAWBACKS AND LINES OF RESEARCH

Since the toxins must be ingested by the target-larvae, it is necessary that the toxin entities, i.e. the crystals or spore/crystals complexes, reach the larval feeding zones and then remain present and active as long as possible to avoid frequent application of larvicides. Depending on the mosquito species, there is a very wide diversity of biotopes in which mosquito larvae can breed (Laird 1988), for example ricefields, ponds, drinking water reservoirs, cesspools, gutters,... Problems encountered for application can be penetration of the covering vegetation, accessibility of the water surface, competition of ingestion between toxin particles and other particles present in water such as plankton, presence of polluants, ... Numerous kinds of experimental formulations have been designed to fulfill these criteria. However, at present the short-term persistance of *Bti* in the field remains the main problem, mainly due to sedimentation of the crystals out of the larval feeding zone or to alteration of the toxins in water.

Some strains of *B. sphaericus* are very active in the laboratory against several malaria vectors such as of *Anopheles gambiae* (Nicolas et al. 1987b), *Anopheles quadrimaculatus, Anopheles albimanus* (Lacey et al. 1988) or *Anopheles stephensi* (Thiery & de Barjac 1989). However, in the natural environment, these mosquitoes generally breed in habitats that are exposed to sun light and field experiments have revealed a short-term efficacy of *B. sphaericus* due to a rapid decay of toxicity caused by UV radiation (Davidson et al. 1984, Lacey & Smittle 1985, Nicolas et al. 1987b). This was also the case in treatment of *Culex pipiens* larvae in lagoons in South of France were recolonization of the lagoon occurred after 1-2 weeks after a treatment at 1 l/ha (Sinègre 1990).

Four lines of research are followed at present to encounter these problems, including improvment of formulations, research of new entomopathogenic strains, modification of the host-range by genetic engineering and expression of toxin genes in microorganisms which naturally colonize the breeding sites and serve as food for the larvae.

Formulations

Long-term efficacy of *B. sphaericus* treatment against *Culex quinquefasciatus* were reported in Ivory Coast and in Tanzania, where larvae were controlled respectively for 5 and 8 weeks in cesspools and gutters with a single treatment of BSP1 at 10 g/m^2 (Hougard & Nicolas 1986, Nicolas et al. 1987a). This formulation allowed a slow sedimentation of the spores in the breeding sites; a threshold of 100 to 500 spore-crystal complexes/ml in the larval feeding zones was necessary to control hatching larvae after the treatment (Nicolas et al 1987a). Despite the high dose of larvicide used in these studies, control of *Culex quinquefasciatus* with *B. sphaericus* was more cost-effective than treatments with chlorpyrifos repeated every two weeks, when expenses of gasoline, vehicles and manpower were taken into account (Hougard & Nicolas 1986).

Significant advances in adjuvants of formulations have been reported by the group of the Lee County Mosquito District in Florida, USA. Good dispersal of *Bti* and *B. sphaericus* at the water surface were obtained with Arosurf® MSF (monomolecular surface film) (Levy et al 1984, 1986). The recent development by this group of a controlled-release system of Bti and *B. sphaericus*, Culigel®, was reported (Levy et al. 1990). This system uses crosslinked modified polyacrylamide granules to entrap the bacteria. Control in a variety of aquatic environments of *Aedes, Culex* and *Psorophora* species for a few months at application rates of 2-3 lbs insecticides granules/acre of water were reported. Moreover, protection of the larvicidal activity of Culigel® granules from loss due to alternate floodings and drying and to photodegradation was reported. Therefore, this kind of formulations can be applied in pretreatments in dry habitats, before flooding.

Efficacy of more than 50 days in drinking water colonized by *Aedes aegypti* in Indonesia, where hemorragic dengue is endemic, has been obtained with tablets allowing slow-release of *Bti* (Becker 1990).

An other approach, the CellCap® system based on genetic engineering has been developed by Mycogen Inc. in California for *B. thuringiensis* toxins of agricultural importance (Barnes et al. 1986, Gelertner, 1990). A toxin gene was expressed in *Pseudomonas fluorescens* cells and recombinant cells were then killed by heat and lugol. This technique provides to the toxin a natural encapsulation. Such idea could be also used for expression of mosquitocidal toxin genes.

Finally, a potential approach could be the expression of the gas vesicle genes of cyanobacteria in *Bti* or in *B. sphaericus* in the hope to get synthesis of gas vesicles and therefore a better flottation of the recombinant cells.

RESEARCH OF NEW ENTOMOPATHOGENIC STRAINS

Beside *Bti*, the most *B. thuringiensis* strain pathogenic to Diptera studied until now is *B. thuringiensis* subsp. *morrisoni* PG14 (serotype H8a8b), isolated from the Philippines (Padua et al. 1982). This strain has the same host-range as *Bti*, a similar toxin composition, except a supplementary protein of 144 kDa (Ibarra & Federici 1986), and great homology in the toxin sequences with *Bti* (reviewed by Höfte & Whiteley 1989). Field trials are carried out, mainly in the Philippines, with this isolate (Padua 1988).

Several strains of *B. thuringiensis* have demonstrated activity to both Diptera and Lepidoptera, such as *B. kurstaki* HD-1, *B.t. galleriae* HD-29 and *B.t. aizawai* IC1 (reviewed by Ellar 1990), but are not competitive with *Bti* or *B. thuringiensis* subsp. *morrisoni* PG14 as microbial insecticides against mosquitoes.

The most active strains of *B. sphaericus* against mosquitoes belong to serotypes H5, H6 and H25 (Thiery & de Barjac 1989). However sequence comparisons between five highly toxinogenic strains of *B. sphaericus* show that the two toxin genes from this organism are very conserved from one strain to the other (Berry et al. 1989).

While it is still worth screening new isolates among *B. thuringiensis* or *B. sphaericus*, in particular from endemic countries, for their toxicity to vectors, there is a great need to look for new biological control agents, in particular bacteria synthetizing toxins which differ in structure and insecticidal mode of action from the toxins of *B. thuringiensis* or *B. sphaericus*.

Recently, a strain of *Clostridium bifermentans*, serovar *malaysia (Cbm)* was isolated at the Institut Pasteur from a mixed bacterial sample originating from Malaysia and was shown to be highly toxic to *Culex pipiens, Anopheles stephensi, Aedes aegypti* and *Simulium damnosum* (de Barjac et al. 1990). Safety tests on mice and golden fishes have shown that innocuity of *Cbm* to these organisms (de Barjac et al. 1990). The larvicidal activity is due to the cells and occurs with the sporulation (de Barjac et al 1990, Charles et al. 1990). It has also been shown that the larvicidal activity decreases when the cell lyses, due to inactivation of the toxicity by extracellular proteinases produced by the bacterium.

This is the first description of an anaerobic bacterium toxic to mosquitoes and blackflies and this opens the field to isolate new toxins.

Modification of host-range by genetic engineering

Two strategies are being used to construct new strains from natural isolates. The first one uses non-recombinant genetic methods and has been used by the industry, for example Ecogen Inc., to develop *B. thuringiensis* strains exhibiting modified host-range against crop pests (Carlton 1990). The first is the isolation of strains presenting different host-range and evidence of the plasmid localization of the corresponding toxin genes. Then curing of plasmids which do not harbour the toxin genes can be performed to enhance expression of these genes. The last step is the conjugal transfer of two plasmids encoding different activities in the same recipient strain. One advantage of this approach concerns legislation. In the USA, these strains are considered as natural unmodified strains and therefore can be developed in 2 to 4 years. No significant result has been reported in vector control using this strategy.

The other approach is DNA recombinant technology. Most of the genes encoding toxins pathogenic for Diptera have now been cloned and sequenced (Höfte & Whiteley 1989, Berry et al. 1989) and the respective role of several toxins has been elucidated (Delécluse et al. 1988, Broadwell et al. 1990, Berry 1990). A recent study (Widner & Whiteley 1990) on the cryIIA (P2) protein of *B. thuringiensis* subsp. *kurstaki* HD-1, which is active against both *A. aegypti* and the Lepidoptera *Manduca sexta*, has determined the amino acid sequence of the protein responsible for mosquitocidal activity. Moreover, by recombination, they have transferred the mosquitocidal activity to cryIIB, an other toxin of HD1, specific of *M. sexta*. Chen et al. (1990) have obtained, by cloning, protoplast fusion and conjugaison, recombinant cells of *Bti* expressing the 135 kDa toxin gene of *B. thuringiensis* subsp. *kurstaki*, and being toxic to both *Anopheles* sp. and *Bombyx mori* (Lepidoptera), at specific activity comparable to parent strains. Extension of the host-range of *B. sphaericus* to *A. aegypti* has been reported by Trisrisook et al. (1990) who expressed the 135 kDa toxin gene of *Bti* into *B. sphaericus* 1593 and 2362. Expression of the *B. sphaericus* toxin genes in *Bti* has also be obtained by Bourgouin et al. (1990), by electroporation of the *Bti* cells with a plasmid harbouring the *B. sphaericus* toxin genes.

Cloning of toxin genes in microorganisms naturally present in the breeding sites

Construction of transgenic plants resistant to attacks by insects, by expression of entomopathogenic toxins in their tissue, has raised a great interest for crop protection (reviewed by Van Mellaert et al. 1988, Perlak et al. 1988). A similar approach has been thought for controlling mosquito larvae, by introducing toxin genes in microorganisms able to propagate competitively in the breeding sites and known to serve as food for the larvae. Few data are available on the food diet of mosquito in the natural environment. Most of the studies examining the natural food of mosquito larvae were conducted more than 50 years ago and were not accurate. Walker et al. (1988) have analyzed the gut content of *Aedes triseriatus, Anopheles quadrimaculatus* and *Coquillettidia perturbans* in Michigan, using a staining method to quantify the different kinds of microorgnisms.

Among the microorganisms present in many mosquito breeding sites, cyanobacteria seem to be good candidates. These photosynthetic organisms are widely distributes in mosquito habitats (Laird 1988). The recently developed genetic studies render feasible the transfer of heterologous DNA and its expression in several strains of cyanobacteria (Tandeau de Marsac & Houmard 1987). Cloning and expression of the toxin genes of *B. sphaericus* 1593 in the unicellular cyanobacterium *Synechococcus* PCC 7942 has been successfully achieved by Tandeau de Marsac et al. (1987). The recombinant clones were highly toxic to *Culex pipiens* larvae. Introduction of the 130 kDa toxin gene of *Bti* has also been reported by Angsuthanasombat & Panyim (1989) in *Agmenellum quadruplicatum* but the level of expression was very low. However, in order to make this approach feasible, several criteria must be fulfilled. Potential cyanobacterial host strains should preferably be indigenous, at least in a given geographic area, to maximize the chance of recombinant clones to survive after reintroduction in the environment. These strains need to propagate competitively with other microorganisms and should be tolerant to seasonal variations of the biotopes. Finally, the cells must be ingested and rapidly digested by the larvae to allow the release of toxins in the gut. In order to find such strains, a study of the cyanobacterial strains present in mosquito breeding sites in the South of France has been set up at the Institut Pasteur (Nicolas et al. 1989). Although this approach is very attractive, a key question to be answered before thinking of application is the potential development of resistance of mosquito larvae to the toxins permanently present in the water.

CONCLUSIONS

The development of bacteriological insecticides has been greatly favoured by the innocuity of these microorganisms to human populations and to the environment. Several programs have demonstrated the feasibility of these agents in mosquito or blackfly control programs when the willingness of using safe insecticides and developing vector or nuisance control strategies were effective. The example of the Onchocerciasis Control Program in West Africa shows that the use of biological control agents is not limited to developed countries, as soon as a structure of vector control is set up. Therefore, there is a great hope to increase the use of these agents in tropical countries where vector-borne diseases occur, with the development of biotechnology in these countries.

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