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Microbiological Control of Dangerous Infections Vectors

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Environment protection initiated the development of biological, i.e. natural means for pest control. The reorientation towards biological means for plant protection has been realized in many countries and microbiological methods are of a crucial importance for pests control. Within the target insects the vectors of dangerous infections have essential importance. The efforts in this area are directed to development of microbiological pest control, namely in the use of insecticides from *Bacillus thuringiensis (**Bt**)* [1-3].

The Scientific Center of Anti-infectious Drugs, **SCAID** in Kazakhstan and microbiological institutions of NAS RA are for many years actively cooperating for search and use of intercalinated by iodine drugs and their use in treatment of various infectious diseases, particularly caused by multiresistant to antibiotics strains with special emphasis for obtaining of nanostructured complexes. During the last 5 years **SCAID** is cooperating with Microbial Depository Center (**MDC)** in studies of biologically active compounds produced by crystalline inclusions forming entomopathogenic bacilli.

The great progress in molecular biology, biochemistry and genetics of ***Bt*** resulted in genetic modifications and obtaining transgenic corn, cotton, tobacco, tomato, potato and other crops resistant to several dangerous pests. Almost all genetic manipulations mostly developed on ***Bt*** at present are available for broad practical application.

Entomopathogenic sporeforming bacteria are represented by a great diversity of species. Undoubtedly the entomopathogenic lead of the Genus *Bacillus* is ***Bt*** first described by E.Berliner in 1915. Essentially important contribution in this field was establishment of direct relationship of insecticidal action of ***Bt*** cultures with formation of crystalline inclusions of proteinous nature during the sporulation of bacteria [4].

The list of entomopathogenic bacilli includes a great bacterial diversity. First of all up to hundred serotypes/subspecies of ***Bt*** with insecticidal activity to many pests. Among other entomopathogens are *B.sphaericus* (valid name *Lysinibacillus sphaericus*, ***Ls***) [5], *B.laterosporus* and many others, including hardly cultivated *B.popilliae/lentimorbus* [2].

Among the ***Bt*** cultures are very pathogenic strains to honey bee and silkworm larvae as well as to mosquitoes, mites, etc. Very important to underline the presence of specificity of action to certain subspecies, serotypes and other taxons.

Till now ***Bt*** keeps since 1959 the leadership in large-scale production of bioinsecticides in the world. In the FSU *Bt* preparations have been produced in limits of 6-8 thousands tons annually. ***Bt*** till now keeps the world leadership in this field.

Microbiological insect control comprises the use of bacteria, fungi, viruses, protozoa and microscopic nematodes. In spite of substantial progress in the study and application of organisms mentioned the leading groups of microorganisms for insect control are represented by some aerobic spore-forming bacteria, particularly ***Bt*** what is the basis of contemporary large-scale industrial production of bioinsecticides against lepidopterean, coleopterean, mosquitoes, black flies, ticks and other pests.

Nevertheless among bacilli there are many other species to be studied and used as perspective source of new insecticides: many subspecies / serovars of *Bacillus sphaericus* (valid name: *Lysinibacillus sphaericus (**Ls**)* [5], *Bacillus popilliae*, *Bacillus lentimorbus*, *Bacillus laterosporus* and related *bacilli* producing specific entomocidal crystalline toxins of proteinous nature and having infectious action on the insect. In comparison with other microorganisms, mainly bacteria and fungi, the advantages of entomopathogenic *bacilli* are that due to formation of spores they are able to resist the unfavorable conditions of environment and to reproduce in nature causing wide-spread epizootics. The Genus *Bacillus* has the priority.

Study and use of bacterial insecticides cover a broad spectrum of R&D comprising many microbiological, biochemical, genetical, technological and entomological topics. In terms of the objectives the scopes have to be limited within the microbiological aspects for screening of new insecticidal mainly non-***Bt*** species of bacilli.

MDC is the successor of long-term R&D of the Institute of Microbiology of NAS RA (**INMIA**), functioned in the Former Soviet Union (**FSU**) as the lead organization in microbiological problems for bacterial pest control. The Collection of entomopathogenic bacilli in **MDC** comprises over 5000 strains including around 1000 cultures of new subspecies, mainly ***Bt, Ls*** and related species.

The Collection includes a lot of extremophilic forms having important practical application for microbiological transformations. Enzymatic Database serves the R&D in this field.

The **MDC** was functioning in long-term R&D for integrated evaluation of microbial deteriorations of synthetic polymeric materials of space technique. The Collection of **MDC** comprises over 1000 fungal biodegradants, included

extremophiles, isolated from inhabited spacecrafts, the Orbital Complex (**OC**) "MIR" and the International Space Station (**ISS**) [6].

At present the Culture Collection kept comprises around 15.000 well-characterized strains, maintained in viable conditions. The Collection is included in the World Federation of Culture Collections (**WFCC**) under the registration number WDCM #803. The strains are kept by systematic transfers in nutritive media, under mineral oil, freeze-dried and in liquid nitrogen.

The **INMIA** organized the large-scale production of two original insecticides: **BIP** - for control of lepidopterean pests and **BLP** - for mosquitoes control.

The great contribution of the **INMIA**'s researchers was the first report on ***Bt*** plasmids and its role in production of entomocidal toxins in 1976 [7].

The main important results of R&D could be summarized as follows:

- Isolation and characterization of new entomopathogenic bacilli for pest control,
- Establishment of cross agglutination between of *B. cereus* and ***Bt*** (Table 1),
- Based on homology of DNA between cultures of ***Bt*** and mosquitocidal strains of ***Ls*** [8], several strains of ***Ls*** have been proposed for testing of cancerolytic action: the tests revealed the presence of this action to cancer culture *in vitro*. The presence of such action with stimulation of interferon has been found out [9],
- Using *B. popilliae'* spheroplasts their fusion with ***Bt*** spheroplasts was succeeded for production of *B. popilliae* recombinants [10].
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Table 1

Serotypisation of B.cereus (Bc) and Bt cultures isolated from different sources

Origin	Spec, Cryst formation	Qn-ty of str	Qn-ty of strains in serotypes					Non. typ.
			berl.	ale.	sotto-dendrolimus	galleriae	caucasicus	
Silk	Bt Cr+ Bc Cr-	70 77	3 0	1 3	5 20	21 1	31 1	9 52
Mulb leaves	Bt Cr+ Bc Cr-	15 4	2	0 0	1 1	4 0	7 0	1 3
Various insects	Bt Cr+ Bc Cr-	18 9	1 0	0 0	5 1	8 0	2 0	2 8
Soil	Bc Cr-	45	0	5	2	2	2	15
Total	Bt Cr+	103	6	1	11	33	40	12
Total	Bc Cr-	135	0	8	24	3	3	78

Molecular genotyping of entomopathogenic bacilli has been applied for identification of different entomopathogenic bacteria mainly ***Bt, Ls*** and related species**.**

The extraction of genomic DNA from bacilli has been done according to benzyl chloride extraction method [11]. DNA samples extracted from the bacilli

have been amplified by PCR technology and visualization of the results obtained has been realized after agarose gel electrophoresis by further exposure under UV transilluminator, what indicates high specificity of used primers ***Bt*** and ***Ls***. Generally, molecular genotyping gives more detailed identification, than serological methods with the use of H-antigen.

Preparation BLP (Bacterial Larvicide Preparation) – the new larvicide bacterial preparation obtained in Armenia and implemented in large-scale production with specific activity to mosquitoes has been successfully used in many regions of **FSU**. The preparation is highly active to mosquitoes of genera *Aedes, Culex, Anopheles, Uranotaenia.* **BLP** is produced on the basis of the original highly active strain of *B. thuringiensis ssp.israelensis.* It can be manufactured in different preparative forms: liquid preparation, paste, dry wetable powder, granules. The larvicidal activity of the dry (powder) preparation is equal to 5000 international units per mg, using *Aedes aegypti*. **BLP** has no toxicity for human, mammals, various hydrobionts (water insects, amphibiae, fishes), useful insects (honey-bee, silkworm) and plants. Its practical application revealed high efficiency in different regions of the **FSU**.

Insecticidal activity of entomopathogenic bacilli to the vectors of dangerous infections

The summarized data on mosquitocidal activity of ***Bt*** belonging to subspecies *israelensis* and ***Ls*** serotypes 5 / 25 to 10 different species of mosquitoes are presented in Table 2.

Table 2

Mosquitocidal activity of entomopathogenic bacilli

Mosquitoes	*Bacillus* species	MDC Nu-s	Mortality of larvae
Aedes caspius Pal., *Anopheles maculipennis* Mg., *Anopheles claviger* Mg., *Culex pipiens* L., *Culex modestrus* Fic., *Culex theileri* Th., *Culex territans* Wal., *Culex hortensis* Fic., *Uranotaenia unguiculata* Ed.	***Bt***	2912, 11953, 11954, 14096, 16363	10-60 min
	Ls	2914, 2977, 2984, 11453	15-24 hrs
Aedes aegypti L.	***Bs***	2922, 2923	10-60 min
	Ls	2914	15-24 hrs

The special series of experiments aimed to characterize insecticidal activity to fleas have been carried out. As a test-organism, larvae of *Xenopsylla cheopis* Roths., the vector transferring the plague has been applied. A special emphasis has been placed on studies of ***Bt*** ssp. *caucasicus* cultures isolated and described by us in previous years as a separate subserovar of serovar 10 (ssp. *darmstadiensis*). The fleas larvae have been selected from generated eggs kept in sterile sand enriched with albumin and yeast extract. For each experiment 30-40 larvae were tested on agar medium. The data are summarized in the Table 3.

The bacillary cultures have been isolated from dominant microbiota of ticks and identified as ***Bt*** species. The obtained bacterial cultures were tested on ixodic ticks in laboratory experiments.

The obtained results permitted to characterize the following cultures as the strains with high insecticidal activity to ticks:

- strains of *Bt* - 14535, 14520, 14572, 14570, 14571, 14575, 15047
- strains of *Ls* – 14522, 14571, 14572, 14574, 14655, 16067.

Table 3

Insecticidal activity of entomopathogenic bacilli of *Bt ssp. caucasicus* to fleas *Xenopsylla cheopis* Roths. *(evaluation of activity of dried biomass 30 mg/g in diet, 20 bln spores/g)*

Strains of *B .thuringiensis*	Age	Mortality, %	Mortality of imago in 6 days, %	LD_{50} of larvae, mg/g
ssp. *caucasicus* 805, 837, 839, 871, 873, 887, 891-896	1	60	50	13
	2	30		15
	3	25		8
ssp. *caucasicus* 811, 828, 831, 844	1	70	80	8
	2	40		12
	3	60		50

The bacillary cultures have been isolated from dominant microbiota of ticks and identified as ***Bt*** species. The obtained bacterial cultures were tested on ixodic ticks in laboratory experiments.

The obtained results permitted to characterize the following cultures as the strains with high insecticidal activity to ticks:

- strains of *Bt* - 14535, 14520, 14572, 14570, 14571, 14575, 15047
- strains of *Ls* – 14522, 14571, 14572, 14574, 14655, 16067.

According to our data molecular genotyping with specific primers gives more and detailed data to reveal biodiversity and identification than other methodologies including seroagglutination. For example, by molecular genotyping it was possible to reveal around 10% of strains identified as non-***Bt*** cultures before they were recognized as ***Bt*** serotypes.

The specific amino acid and vitamin requiring properties of different subspecies of ***Bt*** cultures have been revealed for ***Bt*** (ssp. *berliner*) thiamine, for ***Bt*** ssp. *galleriae* -nicotinic acid and several amino acids.

Our long-term studies have shown that among B. cereus *and related species - the true soil inhabitant without any signs of crystal production, possible to isolate the strains which are serotyped with antiserums of well-known subspecies of* B. thuringiensis.

The obtained data have shown that the cultures of B. cereus *and* **Bt** *possess cross agglutination properties to H-antigen antiserums prepared from original* **Bt** *namely serotype 1 (*berliner*), what indicates phylogenetic closure of these species.*

The combined preparation for control of rodents and fleas has been developed with the use of *Salmonella enteritidis* (rodenticide) and *B. thuringiensis ssp. caucasicus* (insecticide to fleas).

The unique and very important species is *Bacillus popilliae,* which is active to Japanese beetle milky disease and which in certain conditions spontaneously produces spheroplasts. We have already shown that the phenomenon of diauxia blocks the sporulation of *B. popilliae.* This phenomenon was used for obtaining of ***Bt*** recombinants **by** using vitamin requiring properties as genetic markers.

Besides the mentioned species of around 100 strains from the Culture Collection of *Bacillus mycoides*, isolated from soils of different countries, including left-rotating and right-rotating (inversive) forms have been studied for production of crystalline parasporal inclusions and entomocide (insecticide) action to silkworm larvae (4-5 instars).

MDC is actively engaged within the Russian Space Programs **BION, BIORISK, EXPOSE** and **PHOTON.** During last years some strains of *B. mycoides* have been exposed in the **ISS**. The data presented have shown that different forms of *B. mycoides* maintain their entomopathogenic action (test: silkworm larvae). The only difference is the time of larvae's mortality: as usual insecticide activity is revealed after 96 hrs, in contrast to Bt after 24hrs. The forthcoming R&D has to elucidate the nature of this difference in insecticide action.

For many years we have revealed the cultures of *Bacillus mycoides* in cadavers of various insects. Microscopic studies indicate the presence of crystalline inclusions during the sporulation (Fig.1). Tests on silkworm larvae have shown that their feeding by the mentioned bacterial culture resulted in mortality after 2-3 days in contrast to ***Bt*** giving mortality during the first day. Exposure of such strains in the **ISS** confirmed these data.

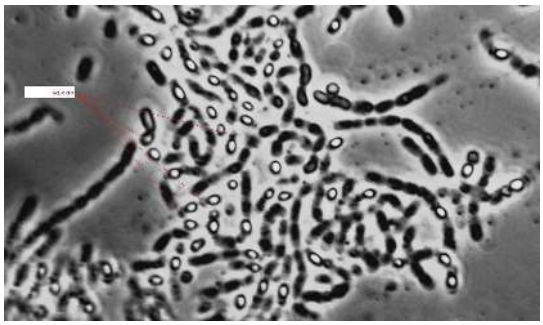

Fig. 1. Phase contrast microscopy of sporulating culture of *B. mycoides* x 1000. The growth after 1 month, the Space **EXPOSE-R** Program

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Микробиологическая борьба с векторами опасных инфекций

Приведен краткий обзор современного состояния микробиологической борьбы с вредоносными насекомыми, в особенности с переносчиками особо опасных инфекций. В результате всестороннего изучения обширной коллекции культур энтомопатогенных бацилл ЦДМ выявлено наличие штаммов с выраженной активностью к комарам, клещам и блохам – переносчикам соответственно малярии, туляремии, энцефалитов, чумы. Установлены перекрестная агглютинация по Н-антигену *Bt* и *B.cereus*, указывающая на их филогенетическое родство, а также образование параспоральных белковых включений у культур *B. mycoides* с сохранением этого свойства после экспозиции и выращивания этих штаммов на Международной космической станции.

Ակադեմիկոս Է. Գ. Աֆրիկյան, Ա. Ի. Իլյին, Ա. Կ. Օկասով

Վտանգավոր ինֆեկցիաներ փոխանցողների դեմ մանրէաբանական պայքարը

Ներկայացվում է կարճ ակնարկ վնասատու միջատների, հատկապես խիստ վտանգավոր ինֆեկցիաներ փոխանցողների դեմ մանրէաբանական պայքարի արդի վիճակը: ՄԱԿ-ի միջատասպան բացիլների կուլտուրաների հավաքածուի բազմակողմանի ուսումնասիրության հիման վրա հաստատվել է մալարիա, տուլյարեմիա, էնցեֆալիտներ, ժանտախտ փոխանցողների՝ համապատասխանաբար մոծակների, տզերի և լվերի նկատմամբ արտահայտված ակտիվությունը: Հաստատված է *Bt* և *B.cereus* H-հակածինով խաչաձևող ագլուտինացիան, որը մատնանշում է նրանց ֆիլոգենետիկ կապը: Հայտնաբերվել են սպիտակուցային պարասպորալ ներառումներ *B. mycoides* կուլտուրաների մոտ, որոնք պահպանվում են Միջազգային տիեզերական կայանում փորձարկումներից հետո:

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Microbiological Control of Dangerous Infections Vectors

A brief review of the state of the Art of Microbial insecticide control activity with emphasis to control of infections vectors has been presented. The comprehensive studies of the vast Culture Collection of entomopathogenic bacilli kept in the MDC permitted to reveal the strains with expressed activity to species of mosquitoes, ticks and fleas as the vectors of malaria, tularemia, encephalitis and plague. The cross agglutination of H-antigen obtained from *Bt* and *B.cereus* has been established, indicating their close phylogenic relationship of the species mentioned. Production of insecticide parasporal proteinous inclusions are distributed in *B.mycoides* strains and preserved during their growth in the International Space Station.

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