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Bacillus thuringiensis STRAINS FROM NATURAL SOURCES IN THE LENINGRAD REGION: ISOLATION AND IDENTIFICATION

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A b s t r a c t

Recently crystal-forming bacilli of *thuringiensis* group are considered the main microbial producers of insecticides. For these bacilli the high adaptability is characteristic leading to wide distribution of these anaerobic spore-forming bacteria in nature. The same *Bacillus thuringiensis* subspecies and variants were isolated on different continents regardless the presence and prevalence or absence of the host insects of this entomopathogen. In different countries and regions the researchers are searching for new *B. thuringiensis* isolates. In the paper the data are represented on *B. thuringiensis* isolation from natural substrates in the territory of Leningrad province. A total of 24 samples of soil, litter, water, silt, sick and died insects have been collected. The samples were cultivated on fish agar. Among more than 3,000 colonies, 62 ones with specific morphology were found. By microscopy with black aniline dye a total of 12 isolates of 62 isolates tested were found out to form both spores and differently shaped crystals of the endotoxin. The microorganisms were selected with regard to entomocidal and larvicidal activity and identified using H. De Barjac, A.A. Bonnefoi (1968) and O. Lysenko (1985) schemes. The investigation made it possible to classify isolates as *B. thuringiensis* of H_1 (var. *thuringiensis*, isolates №№ 12, 20, 40, 41), H_{3a3B} (var. *kurstaki*, isolates №№ 15, 29, 49) and H_{14} (var. *israelensis*, isolates №№ 14, 25, 33, 38, 44) serovars. With regard to biological properties (production of acetyl methyl carbonate, lecithinase, pigment, β-exotoxin; pellicle in broth culture; sucrose, mannose, cellobiose, salicin fermentation; starch degradation; proteolytic activity) these isolates are close to standard strains. Isolates are characterized by high productivity, entomocidal and larvicidal activity and can be used as producers of biologicals against insects and larvae. In the isolates of BtH_1, BtH_{3a3b} and BtH_{14} serovars the titers varied as 2.42×10^9-2.78×10^9; 1.85×10^9-2.15×10^9 and 2.65×10^9-3.28×10^9 CFU/ml, respectively. The activity against *Leptinotarsa decemlineata* Say larvae in isolates №№ 12, 41 of the BtH_1 serovar was the same as in standard strain BtH_1 with LD_{50} at 0.19 %. Entomocidal activity of the isolates №№ 15, 29 and 49 of the BtH_{3a3b} serovar expressed as LD_{50} for *Ephestia kuehniella* of the 2nd instar was 0.88; 0.82 and 0.92 %, respectively, while in the standard strain BtH_{3a3b} the LD_{50} was 0.86 %. In the isolates №№ 33, 44 of the BtH_{14} serovar the titer was the same as in the standard strain, and the activity was even higher compared to the standard. In the isolates №№ 33, 44 the LD_{50} for the 4th instar *Aedes aegypti* larvae was 0.17×10^{-3} and 0.16×10^{-3} %, respectively, when in standard strain BtH_{14} it was 0.18×10^{-3} %. Thus, a total of 12 of the isolates which have been identified as *B. thuringiensis* are close to the type isolates on their biological characteristics and promising as producers of biologics with insecticidal action.

Keywords: *Bacillus thuringiensis*, isolation, identification.

High adaptive capabilities of aerobic spore-forming bacteria *Bacillus thuringiensis* in various extreme conditions are the reason for their wide natural occurrence. The bacilli are reported to be isolated from mountain resources [1]. According to the data of a number of authors [2-6], spore-forming bacteria are widely distributed in soil. The bacillus strains are described that can grow at temperature lower than 45-50 °C, while spores withstand heating up to 102 °C [7].

Earlier, it was considered that the crystal-forming bacteria can be mainly found due to screening sick or dead insects from natural populations. However,

currently it is ascertained that these microorganisms are everywhere, i.e. in soil, water, plants, live and dead insects, forest cover, places of insect habitation [8-13]. Entomopathogens have their habitats related to the migration of host insects. The *B. thuringiensis* bacteria are widely spread in the Crimea where they affected wide range of insects due to favorable local conditions with warn and dry climate. In Asia *B. sotto* and *B. dendrolimus* are isolated, while *B. thuringiensis*, *B. entomocidus* and *B. finitimus* are typical of the USA. In Europe, *B. alesti* strains are typical of the areas where mulberry grows [14, 15]. In recent years, however, the same variants of *B. thuringiensis* were isolated on continents differing by their natural conditions, regardless of the host insect population presence or density [16-19]. Annually, *B. thuringiensis* group is replenished with variants (serotypes) differing not only taxonomically, but also by the range of entomocide efects [20-29]. To date, the scientists from various countries have found and identified over 70 isolates of *B. thuringiensis*. The benefits of these bacteria include their safety for people, homoiothermal animals, useful insects and environment [30, 31].

The purpose of this research was to isolate and identify the bacteria belonging to the *thuringiensis* group, and to select strains prospective as producers of biological preparations with entomocide effect on harmful insects.

Technique. A total of 24 samples were collected from various substrates (soil, forest cover, plant parts, sick and dead insects, water, silt) in St. Petersburg, its suburbs and the Leningrad region.

To isolate microorganisms from insects, a drop of hemolymph or a suspension of tissues taken from sick insects was mixed with a physiological solution and plated on fish agar (FA) in Petri dishes sterilely by an exhaustive smear technique. The same way, inoculation was performed from other substrates (soil, foliage, etc.). After incubation at 28-30 °C for 7 days the cultures capable of forming crystal endotoxin were identified by microscopy of smears using black aniline dye [32].

The isolates were preliminarily screened for entomocidal and larvicidal activity, and the selected variants were identified using the schemes for *B. thuringiensis* (Bt) offered by H. De Barjac, A.A. Bonnefoi [33] and O. Lysenko [34].

To study the biochemical properties of isolates, instead of liquid differential diagnostic media the indicator paper disks were used (Microgen, Russia) which contain certain substrate amounts in combination with the respective indicator stabilized with film-forming polyvinyl alcohol. To determine the ability to utilize hydrocarbons, daily agar culture (in the amount of one microbiological loop) grown at 29±1 °C was suspended in 0.3 ml of sterile 0.85 % NaCl solution (pH 7.3±0.1) and then a disk with a hydrocarbon was placed into the test tube. The disks in the sterile 0.85 % NaCl solution were used as a control. The results were recorded in 5-18 hours. Similarly, paper indicator disks were used to assess the indole production, urease activity, and the production of hydrogen sulphide and acetyl methyl carbinol (AMC).

The bacterial yield was estimated on yeast-polysaccharide media in deep culture using Erlenmeyer flasks for 72 hour incubation at 28 °C on a shaker with aeration (220 rev/min). The cell titer was determined by common technique of serial dilutions with FA.

The biological activity of isolates was determined based on the entomopathogen titer causing lethal effect in 50 % of the tested insects which freely ate the inoculated fodder. Several dilutions of liquid culture were prepared that caused death of 10 to 96 % of the insects. Each variant was tested in three replications, and in the control the fodder was not inoculated.

To assess the biosynthesis of thermostable exotoxin, the liquid culture of

the isolate was centrifuged for 15 minutes at 8,000 rev/min. The supernatant was autoclaved at 105 °C for 20 minutes. Dry milk water suspension (2.5 %, 11 ml), 7 g of wheat bran and 2 ml of supernatant (exotoxin), or 2 ml of sterile water (in the control), were placed into glass vessels of 200 ml. Each vessel contained 20 g of substrate (fodder) and 2 ml of supernatant (0.1 ml/g, or 100 μl/g). The supernatant was used undiluted and diluted at 1:2, 1:4, 1:8, 1:16, and 1:32 corresponding to 50.0, 25.0, 12.5, 6.25, and 3.125 μl of exotoxin per gram. The *Musca domestica*, 3-day-old larvae, 25 insects per vessel, were placed on the substrate. The vessels were kept at 28 °C, and in 5 days the puparia were picked out. The flies that flew out were counted, and the percent of flies that died (X), with an adjustment for those in the control, was calculated by the W.S. Abbot's formula [35]:

$$X = \frac{K - B}{K} \times 100\,\%,$$

where K and B are the number of flies that flew out in the control and tested samples. LD_{50} expressed as the amount of exotoxin in microliters per 1 g of fodder was calculated by the Kerber formula [36].

The entomicidal activity was assessed on 2-day-old larvae of the Colorado potato beetle *Leptinotarsa decemlineata* Say. A water suspension of the bacterial culture liquid (CL) was diluted at 1:10, 1:50, and 1:250 corresponding to the content of 10 %, 2 %, and 0.4 %. A potato branch with five leaves was treated on the two sides with the bacterial culture in the appropriate dilution (vs. water in the control), and put into a vial with water, and then the vials were placed at the angle of 45 into the crystallizer with filter paper at the bottom. Using a brush, 25 larvae were placed on each branch. The vessels were left at room temperature (22-25 °C) for 3 days, after which the fodder was replaced with fresh fodder (untreated). The dead larvae were counted on day 7. The death rate was calculated by the W.S. Abbot's formula for each dilution as adjusted for the death rate in the control [35]. LD_{50} was calculated as to the Kerber formula [36].

When determining the sensitivity of meal moth caterpillars *Ephestia kuehniella* to the isolates, liquid bacterial culture was tested. In this, 2 ml of the dilution (1.0, 0.5, and 0.25 %) was poured into glass vessels of 200 ml with 5 g of wheat flour and then 2-day-old caterpillars, 25 insects per vessel, were placed there and kept at 26 °C with the death rate recorded on day 10. LD_{50} was calculated using the Kerber formula [36].

The larvicidal activity of isolates was assessed in accordance to the World Health Organization recommendation [37] on the 4-day-old *Aedes aegypti* larvae of the insectary population. The CL suspension was prepared by 200-, 400-, 800-, and 1,600-fold dilutions with tap water, which corresponds to the conditional CL content of 0.5×10^{-3}; 0.25×10^{-3}; 0.125×10^{-3}; 0.0625×10^{-3} %, or 5.0; 2.5; 1.25; 0.625 μl of CL per liter. An aliquot of 50 ml of the dilution was poured into Petri dishes and then 25 mosquito larvae were placed into each. The dishes were kept in thermostat for 24 hours at 28-30 °C after which the larva death rate was recorded. The death rate for each concentration, with an adjustment for that in the control, was calculated by the formula:

$$X = \frac{M_o - M_K}{100 - M_K} \times 100\,\%,$$

where M_o and M_K are the arithmetic mean of dead species in the tested and control variants, respectively. Based on the obtained data, LD_{50} was calculated in per cent of the larva death rate using the Kerber formula [20]:

$$\lg LD_{50} = \lg C_M - \sigma\,(\sum X_2 - 0{,}5),$$

where C_M is the maximum tested content; σ is the logarithm of ratio of each previous dilution to the next one (dilution factor logarithm); $\sum X_2$ are the ratios between the dead insects and the total number of those for the dilution.

The obtained data were processed using the dispersion analysis method [38] with a confidence interval of 95 %.

Results. On FA, 62 colonies were selected out of 3,000 ones based on characteristic morphological features of *B. thuringiensis* (color, type and consistence). The microscopy revealed endotoxin crystals of various shapes, along with spores, in 12 isolates of 62 those tested.

The obtained isolates were the bacilli with peritrichous flagellation. These are gram-positive facultative anaerobes. They formed vegetative cells (single or forming short chains of 2-4 cells) of 2.5×0.9 μm in size, and grew well on solid media such as meat peptone agar (MPA) and potato agar (PA). The optimum growth temperature was 28-30 °C. After 48 hours, flat gray-white colonies of round or irregular shape, fine-grained or rough, of viscous consistency, were formed on agar without any changes in the color of the nutrient medium. The cells contained oval spores of 1.1-1.3×0.8-0.9 μm (length×width) and crystalline endotoxin (crystal) located subterminally. In isolates №№ 12, 20, 40, and 41, the crystal of 1.1-1.3×0.9-1.2 μm (length×width) was a regular rhombus in shape with blunt ends and distinct edges, isolates №№ 15, 29, and 49 had crystals of 0.9-1.4×0.9-1.3 μm (length×width) with a prolate regular rhombus shape, and isolates №№ 14, 25, 33, 38, and 44 had crystals of irregular shape sized 0.2-1.1×0.1-0.9 μm (length×width).

On physiological, biochemical and serological properties the isolated bacilli were classified as *B. thuringiensis* and grouped into three serovars (BtH_1, BtH_{14} and BtH_{3a3b}) (Table 1).

1. The main physiological and biochemcial properties of the isolates *Bacillus thuringiensis* (Bt) from natural substrates in the Leningrad region

Isolate №	1	2	3	4	5	6	7	8	9	10	11
12	+	+	–	+	+	+	+	+	+	+	+
20	+	+	–	+	+	+	+	+	+	+	+
40	+	+	–	+	+	+	+	+	+	+	+
41	+	+	–	+	+	+	+	+	+	+	+
BtH_1 (standard)	+	+	–	+	+	+	+	+	+	+	+
15	+	+	–	–	–	–	–	+	+	+	+
29	+	+	–	–	–	–	–	+	+	+	+
49	+	+	–	–	–	–	–	+	+	+	+
$BtH_{3a3в}$ (standard)	+	+	–	–	–	–	–	+	+	+	+
14	+	+	–	–	+	–	–	–	–	+	+
25	+	+	–	–	+	–	–	–	–	+	+
33	+	+	–	–	+	–	–	–	–	+	+
38	+	+	–	–	+	–	–	–	–	+	+
44	+	+	–	–	+	–	–	–	–	+	+
BtH_{14} (standard)	+	+	–	–	+	–	–	–	–	+	+

N o t e. 1, 2, 3, 4 — production of acetyl methyl carbinol, lecithinase, pigment, and β-exotoxin, respectively; 5 — film on beef-extract broth (BEB); 6, 7, 8, 9 — utilization of saccharose, mannose, cellobiose, and salicin, respectively; 10 — starch decomposition; 11 — beef-extract gelatin (BEG) proteolysis; BtH_1, $BtH_{3a3в}$, BtH_{14} — serovars; "+" and "–" — manifestation or no manifestation of trait. The data were processed by dispersion analysis with a confidence interval of 95 %.

To the *B. thuringiensis* var. *thuringiensis* BtH_1 serovar the authors referred isolates №№ 12, 20, 40, and 41 that utilize peptone, meat and fish broth, and protein-rich yeast as nitrogen sources, decompose glucose, mannose, levulose, saccharose, maltose, cellobiose, glycerine, and salicin with acid produced, and do not utilize galactose, arabinose, xylose, rhamnose, lactose, raffinose, mannitol, dulcite, sorbite, inulin, and inosite. These isolates decomposed gelatine, peptonized milk, hydrolized starch, utilized citrates, and produced acetyl methyl carbinol, with no pigments or urease formed. They did not utilze indole or hydrogen

sulphide, and reduced nitrates to nitrites. They were positive in flagellar antigen test with standard antiserum at 1:6400 as *B. thuringiensis* var. *thuringiensis* BtH_1.

Isolates №№ 15, 29, and 49 were classified as the *Bacillus thuringiensis* var. *kurstaki* BtH_{3a3b} serovar. They utilized peptone, meat and fish broth, and protein-rich yeast as nitrogen sources, decomposed glucose, levulose, maltose, cellobiose, glycerine, and salicin with acid production; they did not utilize galactose, arabinose, xylose, mannose, saccharose, rhamnose, lactose, raffinose, mannitol, dulcite, sorbite, inulin, and inosite; and they decomposed gelatine, peptonized milk, hydrolized starch, and utilized nitrates. They synthesized acetyl methyl carbinol, with no pigments or urease synthesized. They did not utilze indole or hydrogen sulphide, and reduced nitrates to nitrites. They were positive in flagellar antigen test with standard antiserum at 1:6400 as *B. thuringiensis* var. *kurstaki*.

The *Bacillus thuringiensis* var. *israelensis* BtH_{14} serovar included isolates №№ 14, 25, 33, 38, and 44. They utilized peptone, meat and fish broth, and protein-rich yeast as nitrogen sources, fermented glucose, maltose, levulose, trehalose, glycerine; they did not ferment saccharose, xylose, lactose, arabinose, galactose, rhamnose, raffinose, mannose, dulcite, sorbite, mannit, inulin, or salicin. They did not assimilate cellulose, or decompose esculin, or release hydrogen sulphide. They produced ammonia, acetyl methyl carbinol, and lecithinase. They reduced nitrates, decomposed gelatine, peptonized milk, and discolored lacmus. Based on the test with standard antiserum diluted at 1:6400, they were classified as *B. thuringiensis* var. *israelensis* (BtH_{14}). They produced crystal endotoxine of an irregular shape, and did not produce exotoxine.

The obtained data (Table 2) evidence a high workability of the BtH_1 isolates. By their biological characteristics, the isolates № 12 and № 41 were not inferior to standard BtH_1.

The results of assessing the productivity and larvicidal activity for the mosquito larvae of the BtH_{14} isolates (Table 3) also indicate a high workability of the isolates. The isolates № 33 and № 44 that are not inferior to standard by the spore titers of and LD_{50} for mosquito larvae are of special significance.

2. Biological characteristics of *Bacillus thuringiensis* var. *thuringiensis* BtH_1 isolated from natural substrates in the Leningrad region ($X \pm x$)

Isolate №	Cell titer, $\times 10^9$/ml	Amount of exotoxin (LD_{50} for L_2 of *Musca domestica*), μl/g of fodder	Entomocidal activity (LD_{50} for L_2 of *Leptinotarsa decemlineata* Say), %
12	2.78±0.11	3.4±0.2	0.19±0.04
20	2.58±0.13	4.0±0.2	0.26±0.04
40	2.42±0.14	4.3±0.2	0.30±0.04
41	2.61±0.10	3.8±0.2	0.19±0.04
BtH_1 (standard)	2.68±0.11	3.7±0.2	0.19±0.04

N o t e. The data were processed by dispersion analysis with a confidence interval of 95 %.

3. Biological characteristics of *Bacillus thuringiensis* var. *israelensis* BtH_{14} isolated from natural substrates in the Leningrad region ($X \pm x$)

Isolate №	Spore titer, $\times 10^9$/ml	LD_{50} for L_4 of *Aedes aegypti*, $\times 10^{-3}$ %
14	2.65±0.13	0.25±0.03
25	2.15±0.14	0.23±0.03
33	3.12±0.12	0.17±0.03
38	2.81±0.14	0.24±0.03
44	3.28±0.13	0.16±0.03
BtH_{14} (standard)	3.38±0.14	0.18±0.03

N o t e. The data were processed by dispersion analysis with a confidence interval of 95 %.

The yield in the BtH_{3a3b} isolates (№№ 15, 29, 49) on the yeast-polysaccharide medium varied from 1.85±0.15 to 2.15±0.14 billion spores per ml. The entomocidal activity for isolates №№ 15, 29, and 49 was 0.88±0.04;

0.82±0.04 and 0.92±0.04 %, respectively, vs. the LD_{50} in the reference strain of 0.86±0.04 %.

Thus, our research confirm the established opinion that the entomopathogenic crystal-forming *Bacilli thuringiensis* (Bt) are found everywhere — in soil, water, forest cover, dead insects, and insect habitats. The identification and biotests showed that the isolates referring to the BtH_1, BtH_{14}, and BtH_{3a3b} serovars by their biological properties and practical significance are close to standard strains. Obviously, analytical selection and proper nutrient media and regimes of incubation can help enhance the practically valuable properties of isolated Bt which can be successfully used as producers of biologicals to control the number of harmful insects.

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