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INSECTICIDAL PROPERTIES OF *Bacillus thuringiensis* var. *israelensis*. II. COMPARATIVE MORPHOLOGICAL AND MOLECULAR GENETIC ANALYSIS OF THE CRYSTALLOGENIC AND ACRYSTALLOGENIC STRAINS

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Abstract

Currently, the bacterium *Bacillus thuringiensis* var. *israelensis* represents a key agent for biological protection against dipteran species, which are harmful to livestock and crop production and transmit infectious diseases of economically important animals. The production strains can be obtained by isolation from natural resources, selection of previously used isolates, screening of genetic collections, and genetic or genomic engineering. The issue of preservation and control of practically valuable properties of strains is of high importance. Biologicals are of significant interest due to their substantial advantages over chemical pesticides and are considered in modern agricultural systems as environmentally and socially priority alternatives to agrochemicals. In the present work, we performed the first comprehensive comparative analysis of crystallogenic and acrystallogenic variants of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) isolated after storage of the strain in different modes. For crystallogenic variants, genes encoding the target insecticidal toxins, Cry4 and Cry11, were detected by the polymerase chain reaction (PCR), and it was shown that the acrystallogenic variants are devoid of these genes. It was found that the culture fluid of crystallogenic variants is approximately 7000 times more active against the *Aedes aegypti* larvae than the same of acrystallogenic. The aim of this work was to compare the morphological, biochemical, technological, larvicidal properties of the crystal-forming and acrystallogenic variants of the strains of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) and testing for the presence of genes encoding Cry insecticidal toxins, which are key determinants of virulence. We studied the strains 404 and 87 stored for 28 years by freeze-drying, then 2 years in fish agar (FA) slant tubes with re-inoculation every 6 months; the 7-1/23 strain stored for 28 years in crystals of NaCl, then 2 years in culture liquid (CL) at 3 °C. Bacterial strains were inoculated on Petri dishes to obtain separate colonies. On day 7 of growth, the 404/14, 87/21, 7-1/23-4 (crystal-forming) and 404/19, 87/33, 7-1/23-8 (acrystallogenic) variants were selected by microscopic analysis using aniline black dye. The differences in the colony morphology were not revealed: the colonies were flat, opaque, grayish-white, rough, rounded, the structure was fine-grained, and the consistency was viscous. The differences either in the morphology of the vegetative cultures, or in the main biochemical properties (the formation of acetylmethyl carbinol, lecithinase, the use of carbohydrates, the splitting of starch, etc.), or in the titer on the yeast-polysaccharide medium were not shown as well. The productivity of the 404/14, 87/21, 7-1/23-4 and 404/19, 87/33, 7-1/23-8 strains varied from 3.36×10^9 CFU/ml to 4.02×10^9 CFU/ml and from 3.74×10^9 CFU/ml to 4.13×10^9 CFU/ml, respectively. The larvicidal activity of the crystal-forming variants, expressed in LC_{50} for L4 *Aedes aegypti*, was $(0.12\text{-}0.16)\times10^{-3}$ %, while acrystallogenic variants were inactive within the standard dilutions ($\times10^{-3}$ %) 1.0; 0.5; 0.25; 0.125; 0.06. Only their 1 % suspension (7000-fold higher concentration) caused 22-39 % death of the *Aedes* larvae after 24 hours; the same concentration of

active variants resulted in 100 % death in 15 minutes. It was established that cultural liquid of the acrystallogenic variants formed a precipitate and a supernatant layer after 12 hours, while the crystal-forming variants remained suspended. The investigated variants of BtH_{14} were analyzed for the presence of genes encoding insecticidal toxins. The results of the PCR analysis with the Bti-specific primers confirmed the belonging of the both crystal-forming and acrystallogenic variants to BtH_{14}. It has been found that the 404/14, 87/21, 7-1/23-4 strains carry genes encoding the Cry4 and Cry11 insecticidal toxins, while 404/19, 87/33, 7-1/23-8 acrystallogenic variants are devoid of these genes agreeing with the absence of larvicidal activity against *A. aegypti*.

Keywords: *Bacillus thuringiensis*, culture liquid, larvicidal activity, insecticidal toxins, Cry4, Cry11

Blood-sucking mosquitoes and flies of the order *Diptera*, transmitters of dangerous infections in humans and animals, significantly damage animal husbandry. *Bacillus thuringiensis* subsp. *israelensis* (BtH_{14}) is an effective bacterial agent of dipteral larvae control used worldwide. Preparations based on such bacteria are used in wide range of habitat of target insects, not causing their resistance and not rendering negative effect on ecosystem [1]. BtH_{14} is considered as priority bioagent in fighting blood-sucking mosquitoes, the parasites of animals and humans and transmitters of diseases, including anaplasmose in cattle stock. Blood-sucking dipteral species can cause a 20-30 % decrease in milk yields and 20-40 % decrease in body weight gain [2].

In plants, including seeded forages, “green” biologicals against harmful organisms based on bacteria [3-5], actinomycetes [6], entomorphtorales and entomopathogenic nematodes [7, 8] as a factor of optimization of agricultural plant protection and nutrition is also an important trend. One of such bioagents is spore-forming bacteria *Bacillus thuringiensis* (Bt) [9, 10] with a complex of useful features. Bt can protect agricultural crops from phytophagans [11, 12] and plant pathogens [13-15], may stimulate plants growth [16, 17], whilst being safe for humans [18] and useful entomofauna [19, 20]. Bt-based biopreparations take the priority place at the market as the most effective and safe for environment [21-23]. Their active substance is spore-crystalline complex [24, 25], in some cases also the thermostable exotoxin [26, 27], as well as a series of other lesser studied metabolites and protein factors of virulence [28-30].

Optimal profitable preparations are obtained at use of producer strain of high virulence, technological effectiveness, and safety. However, any culture of producers is subjected to population diversity [31, 32]. Long storage, frequent reinoculations on solid agar mediums result in formation of colonies of various morphotypes and lead to slight decrease of virulence [33]. Thus, Bt strains stored for 28 years in crystals of sodium chloride or in lyophilized form, and 10 years (observation term) in cryopreserved state remained 100 % viable, but manifested larvicidal activity at a level of 78-90 % as compared to the initial values.

In presented work we for the first time performed comprehensive analysis of isolated crystallogenic and acrystallogenic variants of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) by polymerase chain reaction (PCR) method at early development stage of culture (18 hours). Genes encoding insecticide toxins Cry4 and Cry11 were identified in studied crystallogenic variants, whereas such genes were not found in acrystallogenic ones. Herewith, variants did not differ by main biochemical properties, culture morphology, and colonies on fish agar (FA), as well as by productive capacity. It was established that cultural fluid (CF) in 12 hours after incubation does not form sediment in crystallogenic variants as opposed to acrystallogenic ones, and is approximately 7000 times more active against *Aedes aegypti* larvae.

The purpose of the study was to compare morphological, biochemical, technological, and larvicidal features and presence of entomocidal toxins Cry in vilurent and avirulent variants of BtH_{14} strains.

Techniques. Strains BtH_{14} 404 and 87 were stored for 28 years in lyophilized state, and afterwards 2 years on fish agar (FA) slant tubes with reinoculation every 6 months; 7-1/23 strain was stored for 28 years in NaCl crystalls, and afterwards for 2 years in its CF at 3 °C. Initial productive capacity was characterized by the following values ($\times 10^9$ CFU/ml): 3.96±0.28; 4.4±0.22; 4.25±0.30; LK_{50} for L_4 *Aedes aegypti* comprised $(0.135\pm0.01)\times10^{-3}$ %; $(0.115\pm0.015)\times10^{-3}$ %; $(0.128\pm0.01)\times10^{-3}$ %, respectively.

BtH_{14} strains were cultured on slant FA at 30 °C until full formation of spores and crystals.

Morphology of BtH_{14} colonies was studied on standard fish agar (FA) in Petri dishes using streak plate method on day 7 of growth at 30 °C. Preparations for light microscopy (microscope Zeiss Axio Imager A2, Carl Zeiss, Germany, immersion lens ×100) were stained with aniline black (Lucar, Russia). Crystallogenic and acrystallogenic variants [34] were selected to analyze for crystalline toxin genes [35].

Technological efficacy of BtH_{14} was assessed in yeast-saccharine medium, after 68-hour submerged growing at 30 °C and 220 rpm aeration. The number of cells plated on FA was determined by standard serial dilutions. LK_{50} (larvicidal activity) for *Aedes aegypti* mosquitoes was assessed as described [33].

Biochemical properties (utilization of carbohydrates, formation of acetyl methyl carbinol, indol, etc.) were studied with indicator disks (Paper-Based Systems for Identification of Microorganisms, Microgen, Russia) according to the attached instruction. Cultures grown on FA for 1 day at 30 °C were collected by microbiological loop and placed into sterile sodium chloride (0.85 %). Biochemical analysis of strains was performed in 5-18 hours.

For genomic DNA extraction of for PCR analysis, bacteria were cultured on standard Luria-Bertani (LB) medium during 16-18 hours at 30 °C. Cells were suspended in Tris-EDTA buffer (1 M Tris-HCl, pH 7.5 + 0.5 M EDTA, pH 8.0) and heated during 10 minutes at 102 °C. Cell debris was removed by sedimentation during 3 minutes at 15000 g. Supernatant fluid containing genomic DNA was transferred to eppendorfs and used for PCR analysis (a DNA amplifier T100, Bio-Rad, USA) [35, 36]. Program for PCR analysis was selected based on the annealing temperature of known primers and size of the amplified DNA fragment. Reaction mix (20 μl) contained 1 μl water solution of bacterial DNA (80 ng DNA), 10 μl of Fermentas DreamTaq green PCR master mix (Thermo Fisher Scientific, USA), and 0.3 μl of each primer, final concentration of 1 pmol/μl). Virulent strain BtH_{10} 56 of *B. thuringiensis* var. *darmstadiensis* group was a negative control. PCR products were analyzed by electrophoresis in 1 % agarose gel with 0.002 % ethydium bromide staining.

Data were processed by dispersion analysis [37] with 95 % confidence interval. Tables provide means (*M*) and standard error of means (±SEM).

Results. After long-term storage, BtH_{14} 404, 87, and 7-1/23 strains were plated on FA in Petri dishes and selected for formation of crystalline endotoxin and spores and for spore formation only.

Variants BtH_{14} 404/14, 87/21, 7-1/23-4 (crystallogenic) and 404/19, 87/33, 7-1/23-8 (acrystallogenic) were used in further study.

Cultures did not differ morphologically. In all variants colonies were rough, flat, matt, and grayish-white, of undular edge, fine-grain structure, and viscous consistence. Light microscopy of 16-hour cultures also did not find any differences [Fig. 1, A]. Besides, 7-day cultures of BtH_{14} forms did not significantly differ by morphological properties (see Fig. 1, B).

Table 1 provides data on larvicidal properties in BtH_{14} variants. Findings

show that technological efficacy of crystallogenic and acrystallogenic variants of BtH_{14} strain was practically similar, accordingly $(3.36\pm0.25\text{-}4.02\pm0.15)\times10^9$ and $(3.74\pm0.19\text{-}4.13\pm0.15)\times10^9$ CFU/ml, respectively.

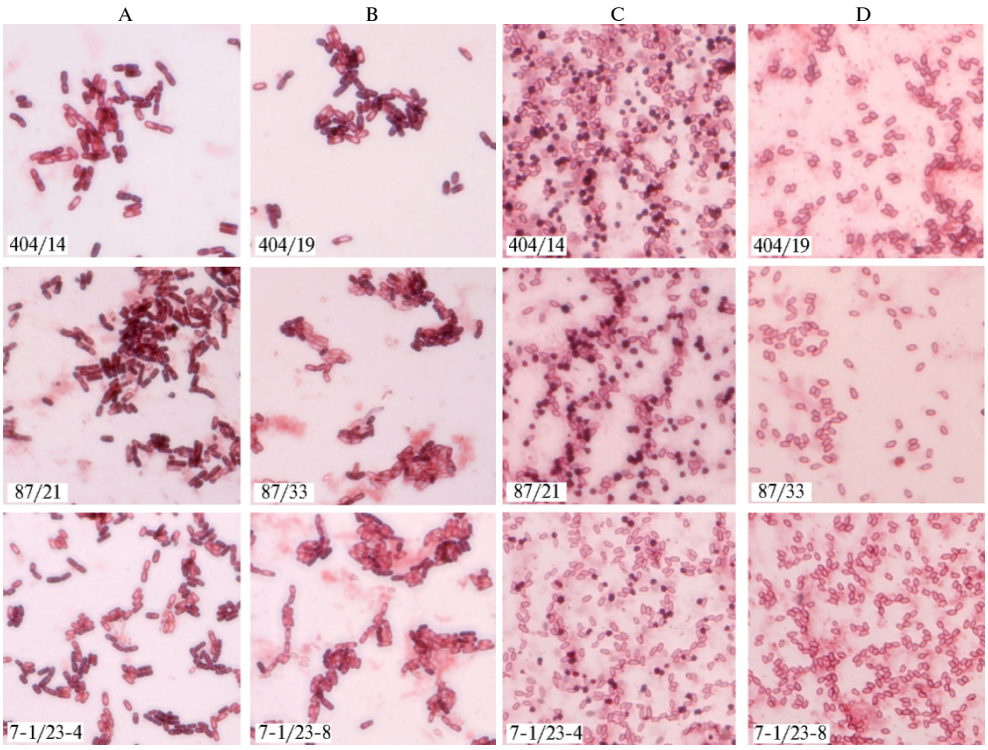

Fig. 1. Crystallogenic (A, C) **and acrystallogenic** (B, D) ***Bacillus thuringiensis* var. *israelensis* (BtH_{14}) variants after 16 hours** (A, B) **and 7 days** (C, D) **of culturing.** Light microscopy (model Zeiss Axio Imager A2, Carl Zeiss, Germany, immersion lens ×100) of preparations stained by aniline black (Lucar, Russia).

Larvicidal activity of crystallogenic variants of BtH_{14} (LK_{50} for L_4 *A. aegypti*) was practically similar within the range $(0.120\pm0.012\text{-}0.160\pm0.018)\times10^{-3}$ %. Acrystallogenic BtH_{14} variants were inactive to L_4 *A. aegypti* in commonly accepted dilutions $(1.0; 0.5; 0.25; 0.125; 0.06)\times10^{-3}$ % CF, and only 1 % suspension (concentration is 7000 times more) caused 22-39 % death in mosquito larvae in 24 hours, whereas in active variants the same concentration caused 100 % death in 15 minutes.

It was interesting that CF of acrystallogenic variants in 12 hours stratified into pellet and supernatant, whilst in crystallogenic variants it remained suspended (Fig. 2). According to our observations, CF of virulent BtH_{14} 7-1/23A strain in suspension preserved for up to 3 years.

1. Larvicidal properties of studied variants of *Bacillus thuringiensis* var *israelensis* (BtH_{14}) ($M\pm$SEM, lab test)

Variant	Results of light microscopy of 7-day culture	Spore titer, $\times10^9$/ml	LC_{50} for L_4 *Aedes aegypti*, $\times10^{-3}$ %
404/14	Spores, crystals	3.36±0.25	0.160±0.018
404/19	Spores	3.74±0.19	0
87/21	Spores, crystals	4.02±0.15	0.120±0.012
87/33	Spores	4.13±0.15	0
7-1/23-4	Spores, crystals	3.95±0.10	0.150±0.020
7-1/23-8	Spores	4.00±0.18	0

N o t e. Results were processed by ANOVA with confidence interval of 95 %. Differences in productive capacity of crystallogenic strains between each other and as compared to acrystallogenic were statistically insignificant. Crystallogenic strains statistically significantly differed from acrystallogenic strains in larvicidal activity.

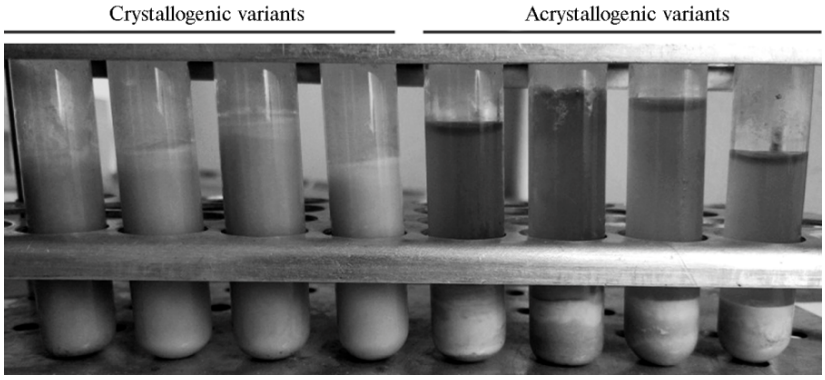

Fig. 2. Culture fluid of crystallogenic *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) variants in 12 hours at room temperature remains a suspension, whereas acrystallogenic variants form pellet.

Crystallogenic (virulent) and acrystallogenic (avirulent) variants of BtH_{14} did not differ in analyzed complex of biological properties. Thus, they have formed acetyl methyl carbinol and lecithinase, hydrolyzed starch, have manifested proteolytic activity and did not have urease activity. Strains metabolized glycerin, glucose, laevulose, maltose, and mannose, did not utilize arabinose, galactose, dulcitol, xilose, salicine, saccharose, sorbitol, cellobiose, and esculite. Moreover, they did not form pigment, but form film on meat peptone broth.

2. Primers used for PCR analysis of crystallogenic and acrystallogenic variants

Primer	Gene	Oligos (5′→3′)	T_m, °C	Reference
Cry11	*cry11*	TTAGAAGATACGCCAGATCAAGC(f) CATTTGTACTTGAAGTTGTAATCCC (r)	45	[38]
Bti		CAAACATTTCATTCCAATAACA (f) ATACTGTGTGGGATGCTTATTA (r)	59	[39]
Cry4	*cry4*	GCATATGATGTAGCGAAACAAGCC(f) ACCTGGAACATCTGACAACCAATC (r)	62	[40] [35]

Since morphologically crystallogenic (virulent) and acrystallogenic (avirulent) variants of BtH_{14} did not demonstrate visual differences, and crystal formation occurs only in few days of incubation, we have characterized crystallogenic and acrystallogenic variants of BtH_{14} strain by PCR using 18-hour cultures. Used primers are presented in Table 2.

Bti primers amplify the nucleotide sequence which is specific for *B. thuringiensis* var. *israelensis* and located on chromosomal DNA. Positive result of amplification with Bti primers (Table 3, Fig. 3) confirmed that the studied variants belong to *B. thuringiensis* var. *israelensis*. PCR analysis revealed *cry4* and *cry11* genes in variants 404/14, 87/21 and 7-1/23-4. In case of variants 404/19, 87/33, 7-1/23-8, no amplification occurred, which confirms absence of crystalline endotoxin and larvicidal activity against *A. aegypti*.

3. Comparative characterization of crystallogenic and acrystallogenic variants of *Bacillus thuringiensis* var. *israelensis* by PCR test

Variant	Primers			Crystallogenicity
	Bti	Cry 4	Cry 11	
404/14	+	+	+	+
404/19	+	–	–	–
87/21	+	+	+	+
87/33	+	–	–	–
7-1/23-4	+	+	+	+
7-1/23-8	+	–	–	–

N o t e. «+» and «–» mean presence and absence of positive reaction or manifestation of the train.

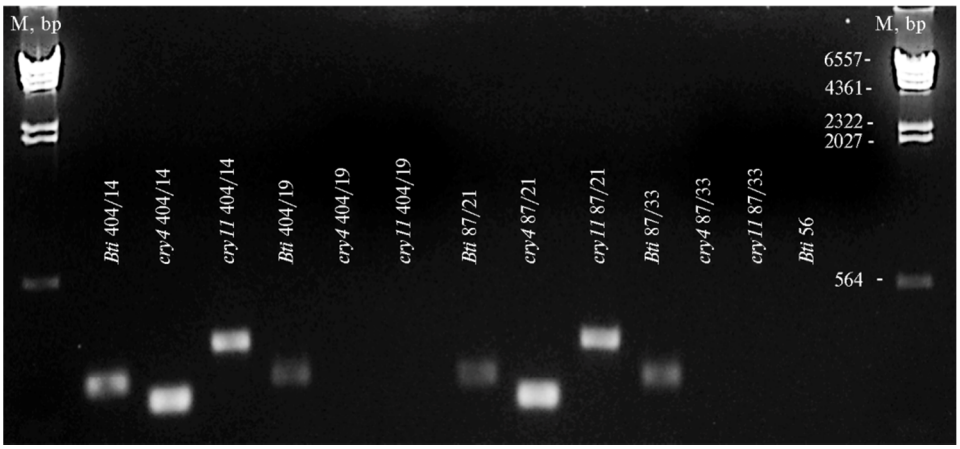

Fig. 3. Gel electrophoresis of PCR products from *Bacillus thuringiensis* var. *israelensis* DNA fragment amplification with Bti, Cry4 and Cry11 primers for crystallogenic and acrystallogenic strains. M — molecular weight marker λ DNA/HindIII (Thermo Fisher Scientific, USA). Genes (as per used primers) and strain numbers are specified.

Therefore, the obtained data draw us to conclude that virulent variants of *Bacillus thuringhighiensis var. israelensis* 404/14, 87/21, 7-1/23, as opposed to avirulent 404/19,87/21, 7-1/23-8, carry genes encoding insecticide toxins Cry4 and Cry11, form crystalline endotoxins, have higher larvicidal activity (1 % suspension of culture fluid of crystallogenic variants caused 100 % death of *A. aegypti* larvae in 15 minutes, whilst acrystallogenic variants caused only 22-39 % death in 24 hours) and remain suspended in culture fluid as opposed to avirulent variants forming pellet in 12 hours. Fast PCR screening to identify genes encoding main protein toxins of *B. thuringiensis* may be helpful in assessing production properties of *B. thuringiensis* var. *israelensis* strains. Since virulent and avirulent variants do not show significant morphological and biochemical differences during long-term culturing, presence or absence of *cry* genes is the most convenient indicator to select promising strains. Molecular genetic methods do not exclude the need for accounting the spore formation rate and ratio of spores to protein crystalline inclusions by light microscopy method, and to control strain titer as indicator of its technological efficacy.

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