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WHOLE GENOME SEQUENCING OF *Bacillus thuringiensis* var. *darmstadiensis* 56 STRAIN AND THE STUDY OF INSECTICIDAL ACTIVITY OF THE BIOLOGICAL PREPARATION ON ITS BASIS

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Abstract

Multifunctional microbiological preparations are promising for use in plant protection due to their diverse effects including growth-promoting effect and complex antifungal and insecticidal activity. One of the key microorganisms used as the basis of biological preparations production is the gram-positive spore-forming bacterium *Bacillus thuringiensis* (Bt). The high specificity of the action and the environmental safety of Bt-based preparations contribute to maintain biocenosis balance and to reduce the number of treatments as well as to obtain environmentally friendly products. Previously, *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) strain was isolated and selected at the All-Russian Research Institute of Agricultural Microbiology. It possesses insecticidal effect for the larval stages of leaf-eating insect pests, growth-promoting activity for potatoes and antifungal effect against various phytopathogenic fungi. This paper presents the first data on sequencing and annotation of the whole genome of the BtH_{10} 56 industrial strain; the factors responsible for the insecticidal and antifungal activity of this strain are identified, and the high efficiency of the biological preparation based on this strain is demonstrated under the field conditions against the Colorado potato beetle (*Leptinotarsa decemlineata* Say.). The goal of the work was to identify the molecular determinants of the insecticidal properties of the industrial strain *Bacillus thuringiensis* var. *darmstadiensis* 56 as well as to test its activity in the field. Field trials of the effectiveness of the biological preparation based on *Bt*H_{10} 56 against the Colorado potato beetle was carried out on potatoes (*Solanum tuberosum* L.) of the Vineta and Rocco varieties in 2018 and 2019 (MTS-Agro LLC, Voronezh Province) in the area of 1 ha. To evaluate the entomocidal activity, we used a liquid form of the preparation based on the strain produced by the Ekos branch of ARRIAM (the spore titer was $2.12\text{-}2.3\times10^9$ CFU/ml) in yeast-polysaccharide medium in a 100 l bioreactor. The application rate of the preparation was 20 l/ha. The potato plantings were treated using an OPG-2000 sprayer (Zarya LLC, Russia). As a chemical standard, the insecticidal preparations Cepellin, EC and Colorado, SC (Agro Expert Group LLC, Russia) at 100 g/l and 0.1 l/ha doses, respectively, were used as the chemical standards. The counts were carried out in 5, 10 and 14 days after treatment. The biological effectiveness of the preparation was determined by analyzing a decrease in the number of pests according to the Abbott formula. According to the test results, the high efficiency of the developed preparation against the Colorado potato beetle was established. This efficiency varied from 83.8 to 87.8 % and did not differ

from the chemical standards. Using Illumina and Oxford Nanopore technology, we obtained the complete genome sequence of the BtH_{10} 56 strain. After assembly and annotation of the genome, a search for toxins was conducted. The CryProcessor and BtToxin_scanner programs were used to search and classify genes encoding the Bt insecticidal toxins. As a result, a gene belonging to the *cry1E* group, *cry1Ea7*, was found. The toxins belonging to this group are characterized by activity against various *Lepidoptera* pests. It was found that the genome of the strain does not contain genes encoding Vip, Sip and Cyt. toxins, however, it harbors several genes encoding synthetases of non-ribosomally synthesized peptides (*nrp*) that may explain its multifunctional properties. Thus, considering the data obtained the liquid form of the biological preparation based on BtH_{10} 56, can be recommended for use in the industry and organic farming.

Keywords: *Bacillus thuringiensis* var. *darmstadiensis*, insecticidal activity, exotoxin, endotoxin, Oxford Nanopore, Illumina, Cry toxin, *Bt*, Colorado potato beetle, *Leptinotarsa decemlineata*

Bacillus thuringiensis (Bt) is a spore-forming soil bacterium widely used as a biological plant protection agent. Currently, about 100 subspecies of this bacterium have been described, isolated all over the world from various sources - insects, soil, plant debris, water reservoirs [1, 2]. Successful commercial products were developed on the basis of the subspecies *kurstaki*, *aizawai*, *san diego*, *tenebrionis* for protection against insect pests [3, 4], as well as the subspecies *israelensis* against bloodsucking dipterans [5)]. Currently, Russian preparations are made only on the basis of two Bt subspecies, *kurstaki* and *thuringiensis* [6].

Biological products based on Bt strains contain a spore-crystalline complex and a number of other metabolites as an active substance. Strains of some varieties during growth and development form and secrete thermostable water-soluble exotoxin (β-exotoxin) into the nutrient medium. The spectrum of action of exotoxin is much wider than that of the spore-crystalline complex [7, 8]. Exotoxin can act not only when infected orally, but also contactly through the covers of insects, and in combination with a spore-crystalline complex can act as a synergist. Bt-based exotoxin-containing preparations are used to reduce the number of *Lepidoptera* insects and also the members of *Coleoptera* and *Diptera* orders. The presence of three main entomocidal components (spores, δ-endotoxin, and β-exotoxin) in the Bt preparation not only enhances entomocidal effect, but also expands the spectrum of action [9, 10].

The subspecies *darmstadiensis* was first isolated in Germany from the larvae of the bee moth *Galleria mellonella* in 1968 [11]. It is known that some of its strains contain insecticidal toxins of the Cry1 group (from “crystal”) that are active against representatives of the order *Lepidoptera*: *Bombyx mori*, *Lambdina fiscellaria*, *Malacosoma disstria*, *Choristoneura fumiferana* [12], *Anticarsia gemmatalis* [13]. Despite the promise of using the *darmstadiensis* subspecies in agriculture and bio-technology, there are no registered commercial preparations based on its strains

Earlier, we screened natural Bt isolates, which resulted in selection of virulent strains of *B. thuringiensis* var. *darmstadiensis* (BtH_{10}). BtH_{10} 56 was isolated from the corpses of the Colorado potato beetle in the Leningrad region, after which multistage selection was carried out for physiological and economically valuable properties [14]. Lab tests showed a high entomocidal activity of the strain against the larval stages of the Colorado potato beetle and potato ladybug, as well as antifungal activity against various plant pathogenic fungi, including *Botrytis cinerea*, *Pythium* spp., *Bipolaris sorokiniana*, *Rhizoctonia solani*, and *Fusarium oxysporum*. In addition, the strain BtH_{10} 56 exhibits a growth-promoting effect, increasing potato green mass and tuber yield, improving the germination of seeds in cabbage, tomato, cucumbers, zucchini, and beets. The growth-promoting effects of BtH_{10} 56 are higher than those of the BtH_{10} prototype

strain No. 25 [15].

Potato is one of the main commercial crops in the Russian Federation. Potato harvest losses caused by its most dangerous pest, the Colorado potato beetle, can reach 40-50% [16]. The need for environmentally friendly products makes biological low-hazard preparations for plant protection all the more attractive. The use of chemical pesticides has a negative effect on the environment and disrupts the ecological connections between organisms. Biological preparations specific in their action are a promising option for plant protection because of their safety for non-targeted biota, the presence of which, in its turn, simplifies the process of keeping the number of pests below the economic threshold of harmfulness. The use of natural regulatory mechanisms along with microbiological control provide environmentally friendly production of foods within sustainable agroecosystems. The biologicals based on the bacterial strain BtH_{10} 56, in addition to pronounced entomocidal activity against the larval stages of leaf-eating pests, has high antifungal activity against various fungal plant pathogens and also has a growth-promoting effect [15]

In the present work, the sequencing and annotation of the complete genome for the producer strain BtH_{10} 56 are performed for the first time, the factors responsible for its insecticidal and antifungal activity are identified, and the high field efficiency of the biopreparation based on this strain against the Colorado potato beetle *Leptinotarsa decemlineata* Say is shown.

The purpose of the work was to identify the molecular determinants of the insecticidal properties of the producer strain *Bacillus thuringiensis* var. *darmstadiensis* 56 and field testing of BTH10 56-based biopreparation.

Materials and methods. Complete sequencing of the BtH_{10} 56 genome was perfumed using Illumina (Illumina, Inc., United States) techniques and monomolecular nanopore sequencing (Oxford Nanopore, UK). NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, USA) was used to construct a genomic DNA library. Sequencing the library (Illumina HiSeq2500, HiSeq Rapid Run v2 sequencing reagents) resulted in 2735262 reads of 250 nt, 683.8 million nt in total. Primer sequences and regions of poor read quality (<q30) were removed with Cutadapt v. 1.17 software [17] and Sickle v. 1.33 (https://github.com/najoshi/sickle), respectively. Additionally, genomic DNA was sequenced on a MinION system (Oxford Nanopore, UK) using Ligation Sequencing kit 1D protocol with FLO-MIN106 cells. The resultant 31,234 reads with an average read length of 16540 nt were generated, 516.6 million nt in total, which were trimmed with Canu v. 1.6 software (parameter –correct) [18]. Then, a hybrid assembly of filtered Illumina reads and corrected MinION reads was performed with SPAdes v. 3.11.1 software [19]. The additionally obtained contigs were once more assembled by npScarf method [20] using MinION-generated raw reads. The gaps between the contigs were filled by consensus sequences from the Illumina reads using the SPAdes graph (––spadesDir parameter of npScarf). The search for genes and their annotation was performed using RAST server (http://rast.theseed.org/FIG/rast.cgi) followed by a comparison of the sequences of the predicted proteins with the NCBI databases. The CryProcessor program (https://lab7.arriam.ru/tools/cry_processor/) was used to search and classify Cry toxin genes, and the BtToxin_scanner program (http://bcam.hzau.edu.cn/BtToxin_scanner/index.php) was used to identify other Bt insecticidal toxins.

In field conditions, the effectiveness of a *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56)-based biological against Colorado potato beetle was as-

sessed on potatoes (*Solanum tuberosum* L.) Vineta variety in 2018 and Rocco variety in 2019 (1 ha, MTS-Agro LLC, Voronezh Province). The tested liquid biopreparation was produced in 100 l fermenters (Ekos branch of ARRIAM), (east-polysaccharide medium, spore titer of 2.12×10^9-2.3×10^9 CFU/ml). The quality of the biological was evaluated by standard methods [21].

An OPG-2000 sprayer (Zarya LLC, Russia), 20 m working width, was used to apply preparations. The biopreparation was applied at 20 l/ha. Insecticidal preparations Cepellin, CE (in 2018) and Colorado, BPK (Agro Expert Group LLC, Russia) (in 2019) at 100 g/l and 0.1 l/ha, respectively, were chemical standards. The pest numbers were counted immediately before and 5 and 10 days after treatment (in 2018 and 2019) and, in addition, 14 days after treatment (in 2018). Five potato plants adjacent to each other were collected diagonally at 20 points (100 plants in total). The biological effectiveness of the biopreparation was determined by the Abbott formula based on the decrease in pest numbers [22].

Results. A total of 6290617 bp was determined for BtH_{10} 56 genome using two technologies (Illumina and monomolecular nanopore sequencing) (Table 1). There were seven contigs in total of which chromosome and two plasmids were assembled as circular contigs; another 4 contigs represented plasmids in a linear form which may be due to the presence of extended repeats.

The *B. thuringiensis* BTH10 56 genome was found to comprise 13 copies of the operon of rRNA genes (16S—23S—5S) and 107 transport RNA genes (tRNAs) encoding all 20 amino acids. As per the annotation, 6611 potential protein-coding genes were predicted, the functions of 4517 (68%) proteins were predicted through comparison with NCBI databases. CRISPR loci (clustered regularly interspaced short palindromic repeats) were not found in the B. *thuringiensis* BTH10 56 genome.

1. Assembly and annotation of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) genome

Cjntig	Structure	Size, bp	Protein-coding genes	tRNA genes	rRNA genes
1	Circular	5553288	5755	107	39
2	Circular	349728	445	–	–
3	Linear	155294	173	–	–
4	Circular	140546	140	–	–
5	Linear	57038	68	–	–
6	Linear	24713	21	–	–
7	Linear	10010	9	–	–
Total		6290617	6611	107	39

N o t e. Dashes mean the absence of the genes.

In the *B. thuringiensis* BtH_{10} 56 genome, there were 6 clusters of *nrp* genes encoding nonribosomal peptide synthetases that could produce various peptides with antifungal and antimicrobial activity [23]. The presence of these sequences in the BtH_{10} 56 genome may explain its antifungal properties.

An insecticidal toxin gene located on one of the large plasmids (contig 3) was identified in the BtH_{10} 56 genome. Analysis of the amino acid sequence of the corresponding protein showed that the toxin belongs to the group Cry1E, subtype Cry1Ea7. Cry1E toxins are three-domain insecticidal toxins of *B. thuringiensis* that are active against various *Lepidoptera* insects [24, 25]. Cry1Ea toxins are characteristic of the *darmstadiensis* subspecies and, as per data published, are active against larvae *Lepidoptera* members *Conopomorpha cramerella*, *Manduca sexta*, *Spodoptera littoralis*, *Bombyx mori*, *Lambdina fiscellaria*, *Malacosoma disstria*, *Cacyreus marshalli*, *Anticarsia gemmatalis*, *Choristoneura fumiferana* [12, 13, 26, 27], which allows the strain BtH_{10} 56 to be deemed promising against these pests.

Cytotoxic proteins Cyt and vegetative toxins Vip are characteristic of some Bt subspecies [8]. However, we did not find the *Cyt* and *Vip* genes in the genome of the studied strain. The insecticidal activity of BtH10 56 against leaf-eating insect larvae, previously detected in lab tests [14], is apparently due to the production of Cry1Ea toxin.

Since an efficiency of a strain in lab tests can significantly differ from its effect on the natural population of insect pests, the next stage of our study was the field tests of the insecticidal activity of the biological.

2. Effectiveness of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56)-based liquid biological against Colorado beetle (Vineta variety, Voronezh Province**,** 2018)

Variant	Pest number				Effectiveness, %		
	before treatment	days after treatment			days after treatment		
		5	10	14	5	10	14
BtH_{10} 56	426	193	52	16	54.7	87.8	96.2
Cepellin (standard)	181	104	27	0	42.5	85.0	100.0
Control (no treatment)	229	254	298	288			

N o t e. Day 10 and day 14 correspond to double treatment.

3. Effectiveness of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56)-based liquid biological against Colorado beetle (Rocco variety, Voronezh Province)

Variant	Pest number			Effectiveness, %	
	before treatment	days after treatment		days after treatment	
		5	10	5	10
BtH_{10} 56	285	145	46	49.1	83.8
Colorado (standard)	171	28	18	83.6	89.5
Control (no treatment)	133	144	186		

N o t e. Day 10 and day 14 correspond to double treatment.

In 2018, surveys on Vineta potato crops prior to the treatment showed 30-40% plants to be populated by Colorado beetle at a 100 m^2 distance from the field edge. Insignificant focal distribution of the pest occurred over the remaining area. The pest population consisted of I (58.5%), II (28.7%) and III (12.8%) larval instars. Immediately after counting, potato plantings were treated with preparations. In 5 days the effectiveness of the BtH_{10} 56-based biological was 54.7%, being slightly higher compared to the chemical standard Cepellin (42.5%). Because of hot and dry weather (air temperature was 37 °C), the potato plants were re-treated. On day 10 after the first treatment (day 5 after the second one), the efficiency was 87.8% being comparable to that of the chemical standard. On day 14, the effectiveness of the BtH_{10} 56-based preparation reached 96.2% (Table 2).

In 2019, the chemical standard for Rocco variety was Colorado insecticide. On day 5, the effectiveness of the biological was 49.1%, being lower than that of the chemical standard. In hot weather (air temperature reached 38-40 °C), the pest developed intensively, so the treatments with BtH_{10} 56 and the chemical standard were repeated. On day 10 after the first application the effectiveness for the biological preparation was 83.8%, for the chemical standard 89.5% (Table 3).

In general, tests of a BtH_{10} 56-based biological preparation (Voronezh Province, 2018-2019) showed its high efficiency against Colorado potato beetle, 83.8-87.8% on day 10 for two different potato varieties. These field data are consistent with the previous lab findings [14]. However, the effects of Bt-based preparations are not limited to the role of an insecticide. There is reason to believe that the growth-promoting effect due to the production of siderophores, indole-3-acetic acid, 1-aminocyclopropane-1-carboxylate deaminase, and en-

zymes that dissolve mineral phosphate is significant [28-30]. The multifunctional properties of the BtH10 56 strain shown by us earlier [14, 15] may indicate this strain to be a promising plant protection agent due to its safety for non-targeted biota, growth-promoting action and antifungal activity. The antifungal effect of a number of Bt strains is associated with the production of short Nrp peptides (nonribosomally synthetized peptides) formed by special synthases via an alternative ribosome-independent pathway bypassing the translation apparatus [31]. Six clusters of such genes we have identified in the BtH_{10} 56 genome.

The insecticidal activity obtained in the field trials is consistent with sequencing and annotation of the BtH_{10} 56 genome, which indicate a gene encoding the Cry1Ea7 toxin. According to the literature, this type of toxin is active against members of order *Lepidoptera* [12, 13, 26, 27]. At the same time, Cry toxin can be effective against different orders of insects, which can be detected only experimentally, as a result of difficult long-term experiments [32]. It is also impossible to exclude the probable influence of other virulence factors on the diversity of insect pests affected by Bt bacteria [8]. In particular, this strain produces thermostable exotoxin [14]; however, we did not find genes for the biosynthesis of class I exotoxin in the BtH_{10} 56 genome. Thus, this strain could produce class II exotoxin. The genetic control of this toxin as not yet been clear, however, it has been previously shown to possess activity against insects of *Coleoptera* order [33]. Probably, the synergistic effect of this exotoxin together with Cry1Ea7 causes a strong toxic effect on the *L. decemlineata* larvae shown in field and lab tests.

At present, *Bacillus thuringiensis* var. *darmstadiensis*-based preparations are not offered in Russian market, however, Baciturin developed in Institute of Microbiology of the National Academy of Sciences of Belarus is successfully used in Belarus (the active substance is the spore-crystalline complex and thermostable β-exotoxin of *Bacillus thuringiensis* var. *darmstadiensis*). Baciturin in field experiments shows similar efficacy against Colorado beetle, 85-94% [34]. In addition to var. *darmstadiensis*, an action against Colorado potato beetle is characteristic of var. *thuringiensis*-based preparaions, for example, Bitoxibacillin® registered in the Russian Federation (LLC PO Sibbiofarm, Berdsk) [6]. Many of the known var. *thuringiensis* and var. *darmstadiensis* strains can produce both endotoxin and β-exotoxin, resulting in similar insecticidal activity of preparations based on these subspecies [35]. In other countries, biologicals based on *B. thuringiensis* var. *aizawai* and var. *tenebrionis* are used to control the Colorado potato beetle, however, only the insecticidal endotoxins Cry1Ia and Cry3Aa are the active components [4, 36].

Thus, the insecticidal activity of *Bacillus thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) is caused by the presence of a gene encoding Cry1Ea7 toxin and also to an exotoxin, probably belonging to class II. Genes encoding protein toxins of the Cyt, Vip and Sip groups, as well as class I exotoxin, are absent in this strain. The presence of genes encoding a series of synthetases of the nonribosomally synthesized Nrp peptides determines antifungal properties of BtH_{10} 56. The results of two-year field trials indicate high entomocidal activity of the liquid form of the BtH_{10} 56-based biological under commercial farming (Voronezh Province) which is comparable to that of the chemical standards. Our data indicate the suitability of using BtH_{10} 56-based biological preparation in integrated plant protection systems and in organic farming.

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