DOI:10.26104/IVK.2019.45.557

ИЗВЕСТИЯ ВУЗОВ КЫРГЫЗСТАНА, № 1, 2019

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КЫРГЫЗСТАНДЫН ТАБИГЫЙ БУЛАКТАРЫНАН БӨЛҮНҮП АЛЫНГАН *BACİLLUS THURİNGİENSİS* ШТАММДАРЫНЫН БИОЛОГИЯЛЫК ЖАНА МОЛЕКУЛЯРДЫК МҮНӨЗДӨМӨСҮ

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БИОЛОГИЧЕСКАЯ И МОЛЕКУЛЯРНАЯ ХАРАКТЕРИСТИКА ШТАММОВ *BACİLLUS THURİNGİENSİS* ВЫДЕЛЕННЫХ ИЗ ЕСТЕСТВЕННЫХ ИСТОЧНИКОВ КЫРГЫЗСТАНА

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BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF NATIVE *BACILLUS THURINGIENSIS* ISOLATES FROM KYRGYZSTAN

УДК: 579.22:632.9

Түндүк Кыргызстандын ар кандай аймактарынан чогултулган топурак жана өлгөн курт кумурскалардын үлгүлөрүнөн Bacillus thuringiensis бактериясынын 15 штаммы бөлүнүп алынды. Жарык жана фазово-контрастык микроскоп астында изилдөө өткөргөндө, бөлүнүп алынган изоляттар өндүргөн параспоралык денечелердин формалары бипирамидалдуу жана куб түрүндө болгону аныкталды. Изоляттар биохимиялык, молекулярдык жана биологиялык скрининтин негизинде мүнөздөлдү. Он беш изоляттардын ичинен сегиз изолят cry гендерине туура келген копияларды камтыганы Cry гендердин плазмидасын коддогон төрт ар кандай түрлөрдүн максатттык амплификациянын натыйжасында аныкталды, жана көпчүлүк штаммдарда cry2, cry4 жана cry3 гендери көп кездешкени аныкталды. Bacillus thuringiensis серотиби galleriae Cry3 гени менен, Bacillus thuringiensis серотиби partial 16S rRNA гени менен, Bacillus thuringiensis Gaoshi-116S Cry2 гени менен, Bacillus sp. B25 Lep2 гени менен, жана ошондой эле Bacillus thuringiensis coreanensis Cry4 гени менен, Bacillus thuringiensis kurstaki Cry4 жана Cry2 гендери менен жана Bacillus thuringiensis серотиби galleriae Cry3 гени менен идентификацияланды.

***Негизги сөздөр:** штамм, биохимиялык мүнөздөмөлөрү, cry гендер, биологиялык коргоо, Bt биопестициддер, параспоралдык кристаллдар, органикалык айыл чарба.*

Пятнадцать изолятов Bacillus thuringiensis были выделены из образцов почвы и погибших насекомых, собранных в различных районах Северного Кыргызстана. Исследование методом световой и фазово-контрастной микроскопии показало наличие бипирамидальных и кубовидных параспоральных тел, продуцируемых этими изолятами. Изоляты были охарактеризованы на основе биохимического и молекулярно-биологического скрининга. Целевая амплификация четырех различных типов кодирующих плазмиду cry генов показала, что восемь штаммов из пятнадцати содержат соответствующие копии генов cry; cry2, cry4 и cry3 гены присутствуют в большинстве штаммов. Были идентифицированы серотип Bacillus thuringiensis galleriae с геном Cry3, серотип Bacillus thuringiensis partial с 16S rRNA геном, Bacillus thuringiensis Gaoshi-116S с Cry2, Bacillus sp. B25 с Lep2 геном, также серотип Bacillus thuringiensis coreanensis с Cry4 геном, Bacillus thuringiensis kurstaki с Cry4 и Cry2 генами и серотип Bacillus thuringiensis galleriae с Cry3 геном.

***Ключевые слова:** штамм, биохимическая характеристика, cry гены, биологическая защита, Bt биопестициды, параспоральные кристаллы, органическое сельское хозяйство.*

Fifteen isolates of Bacillus thuringiensis were isolated from soil samples and dead insect bodies collected in the various districts of North Kyrgyzstan. Light and phase contrast microscopy investigation have showed the presence of bipyramidal and cuboidal parasporal bodies produced by these isolates. The isolates were characterized on the basis of biochemical and molecular biology screening. Biochemical tests included protease (caseinase and gelatinase) amylase fermentation of glucose, sucrose and maltose. Targeted amplification of four different types of plasmid-encoded cry genes has demonstrated that eigh strains from fifteen contain respective cry gene copies; cry2, cry4 and cry3 genes are present in most strains. Bacillus thuringiensis serovar galleriae with Cry3 genes, Bacillus thuringiensis partial 16S rRNA gene and Bacillus thuringiensis strain Gaoshi-1 16S with Cry2 genes, also Bacillus sp. B25 genome with Lep2 genes, Bacillus thuringiensis serovar coreanensis with Cry4 genes and Bacillus thuringiensis serovar kurstaki with Cry4 and Cry2-genes, also Bacillus thuringiensis serovar

DOI:10.26104/IVK.2019.45.557

galleriae with Cry3-genes were identified.

Key words: *strain, biochemical characteristics, Cry genes, biocontrol, Bt biopesticides, parasporal crystals, organic farming.*

Introdtion. *Bacillus thuringiensis* is a Gram-positive bacterium that occurs naturally in soil, water, dead insects and grain dust [1]. *Bacillus thuringiensis* (Bt) is a unique bacterium in that it shares a common place with a number of chemical compounds which are used commercially to control insects important to agriculture and public health. The mechanism of action of Bt toxins on insect pest involves specific molecular interactions which makes Bt a popular choice for pest control. Bt is safe for humans and is the most widely used environmentally compatible biopesticide worldwide [2, 3, 4].

The specificity of action of Bt toxins reduces the concern of adverse effects on non-target species, a concern which remains with chemical insecticides. The greatest successes in microbial pesticides have come from the use of commercial preparations of *Bacillus thuringiensis* (Bt). These have been the most successful biological pest control products worldwide, and 95% of microbial pesticides sold are of this bacterial agent, with annual sales estimated at $100 million [5] . In Kyrgyzstan, there are still intact and unexplored habitats that could serve to detect new species of crystal-forming bacteria and to increase the chance to find new isolates that have insecticidal activity against pests from different orders. The biological properties, diversity, ecology and the occurrence of crystal-forming bacteria in the natural objects of Kyrgyzstan are still untapped and there is only sketchy information about it [6].

Material and Methods. Natural sources for the isolation of BT strains: Soil and dead insect samples were collected in natural landscapes of North Kyrgyzstan as sources for the isolation of Bt species.

Isolation of BT strains from soil. Samples of 10 g were prepared from each soil sample and ground in a sterile porcelain mortar for 5 minutes in aseptic conditions. After grinding, the soil sample was washed in sterile water. To 10 mL of Luria-Bertani broth, 1 g from each soil sample was added and buffered with sodium acetate (0.25 M, pH 6.8) in a 125 mL flask. The broth was incubated in a shaker at 200 rpm for 4 hours (h) at 30°C. A 1 mL aliquot was heated to 80°C for 15 min in a thermomixer (Eppendorf), spread on nutrient agar plates (NA), and incubated at 30°C for 48-72 h. *Bacillus*-like colonies were sub cultured on new NA plates until pure cultures were obtained, and they were kept at 4°C for further identification [7, 8, 9].

Isolation of BT strains from insect samples The surface of the insect's body was previously sterilized in the 700 hydrated alcohols for 2-3 min to avoid contact with foreign microflora, then was flamed in a sprit lamp and immersed in sterile saline solution (NaCL, 0.85%) and repeatedly washed. After that, the bodies were ground in 1 ml of nutrient broth. The broth was incubated in a shaker at 200 rpm for 4 h at 27-28 °C. The homogenate dilutions (10^{-1}, 10^{-4} and 10^{-6}) of each sample were prepared. Of these, 0.1 ml from the last dilutions were plated on the surface of nutrient agar and incubated at 27-28°C.

Phenotypic characterization of BT strains. The conventional tests were performed, such as protein hydrolysis, including reduction of nitrates to nitrites, reduction of nitrates to nitrogen, indole production (tryptophane), fermentation glucose, arginine dihydrolase, urease, hydrolysis (β-glucosidase, esculin), hydrolysis (protease, gelatin), hydrolysis β-galactosidase (para-Nitrophenyl-β-D-galactopyranosidase) and 12 assimilation tests, with substrates such as glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, sorbitol, dulcitol, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid. Phenotypic and biochemical characteristics of isolates were established according to the determinants and manuals.

Amylase Activity. A plate of starch-nutrient agar plate was streaked once with the organism. After incubation for 24 h at 37°C, plates were flooded with 5-10 ml of Lugol`s iodine solution. Any clear area around the growth the culture indicated the breakdown of starch by the organism due to its production of amylase.

Catalase Test. Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide to water and oxygen. The presence of catalase is important

DOI:10.26104/IVK.2019.45.557

in the prevention of toxic by products of oxygen metabolism that can kill the cell.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Isolates were grown on nutrient agar at 37°C overnight. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production.

Hydrolysis of Gelatin. A tube of nutrient gelatin was inoculated by stabbing with a heavy inoculum from 24 h culture and incubated for up to 48 h at 37°C. The culture and an uninoculated tube were stored in a refrigerator for 24 h before checking them for hydrolysis.

Hydrolysis of Casein. Caseinase activity was detected by spotting a very small amount of bacteria onto the center of a dried plate of milk agar. After incubation for 24 h at 37°C, clear zones around the colonies were taken as the evidence for caseinolytic activity.

Fermentation of Carbohydrates. The fermentation of carbohydrates is usually tested by growing the bacteria in peptone water containing 0,5-1% of various carbohydrates (Glucose, Sucrose and Maltose). Acid formation is detected by the addition of an indicator, such as Andrade`s solution. Andrade`s solution is pH 7,2, when it is faintly yellow or colorless; in the presence of acid it becomes red [10].

PCR analysis. Bacterial 16S rRNA genes were amplified by using primers 27F-HT and 1492R-HT. A partial sequence of the pyruvate carboxylase encoding *pycA* gene was sequenced as a molecular-taxonomic marker to provisionally determine the systematic position of the isolates with respect to the *Bacillus cereus* sensu lato complex as part of the multilocus sequence analysis (MLSA) scheme introduced by Priest et al [11]. Screening for cry genes was carried out using the family specific primer pairs directed toward the identification of some of the main groups of *cry* genes.

Results and Discussion

Isolation of BT strains from natural objects. In total, 55 soil samples and 50 insect bodies were collected from different ecosystems of North Kyrgyzstan during 2017-2018. A total of 15 Bt strains were isolated from these natural substrates.

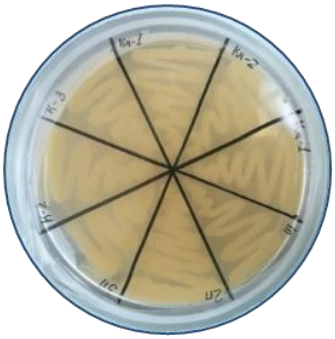
Table 1

Source of Isolation of Bt strains

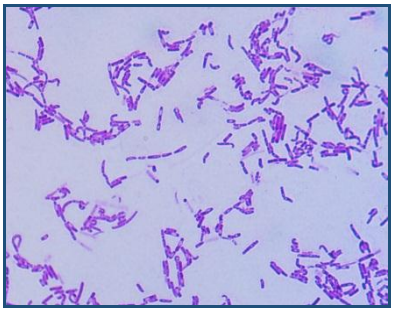
Source of Isolation	Isolates
Soil	1п, 2п, 3п, Б-1, Кж-1, KS, ТК, КТ, OS
Insects	К-2, К-3, Км-1, Км-2, 12К, ВВ, АВ

Phenotypic/physiological and biochemical characterization of Bt isolates.

Crystal Protein Morphology of Bt Isolates. The phenotypic characterization tests have indicated that natural isolated were Gram-positive, spore and crystal forming, motile and catalase was positive. Bacteria were motile and produced ellipsoidal endospores, located at subterminal position in the sporangia, and formed cream-colored colonies with irregular or circular edges on nutrient agar (Fig. 1).



A



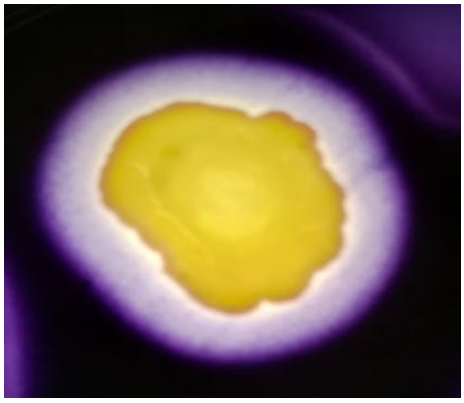
B

Figure 1. Colony morphology of the isolates (A). Phase contrast photograph of the isolate П-2 (B).

DOI:10.26104/IVK.2019.45.557

For the differentiation of Bt strains, we have used a tentative determination key by biochemical reactions for phenotypic characters. These analyses have revealed that all obtained Bt strains do not produce acid from mannose and sucrose for except some serotypes. All strains were able to hydrolyse gelatin, esculin ferric citrate and starch. The ability to hydrolyse cellobiose, lecithin and urea was variable.

All reference strains produced amylase on starch nutrient agar and showed a catalase activity (Fig.2- A and B).



A

B

Figure 2. Amylase activity of isolate Км-2 (A). Catalase activity of isolate Б-1 (B).

All isolates have hydrolyzed gelatin and casein (Fig. 3 - A and B).

A

B

Figure 3. Positive gelatin hydrolysis test (A). Casein hydrolysis test (Км-1 isolate produces a clear zone, a zone of casein hydrolysis) (B).

All strains were positive for maltose fermentation and produced acid in 24 h incubation (Fig. 4).

A

Figure 4. Positive (left) and negative (right) degrade.

DOI:10.26104/IVK.2019.45.557

Cry gene diversity in BT isolates from Kyrgyzstan. The *Cry* toxin contained in the crystal is the virulence factor that truly distinguishes Bt from its genetic cousins Ba and Bc. *Cry* genes are mostly harboured on large plasmids where they often occur in clusters of different cry variants. A large number of *cry* toxin encoding genes have been analyzed and organized into several groups that in part reflect host group adaptation. In particular, proteins encoded by typical *cry1, cry3,* and *cry4* genes are toxic to *Lepidopteran, Coleopteran, and Dipterian* insects, respectively. Targeted amplification of four different types of plasmid-encoded cry genes demonstrates that eighth strains from fifteen contain respective cry gene copies, *cry2, cry4* and *cry3* genes are present in most strains [13] (table. 2).

Table 2

Plasmid-encoded cry genes, relieved in natural Bt strains

Strain	Isolation Source	Gene sequences (5'_3') of primer	PCR Product Size (bp)	Definition of BT strains in GenBank
12-К	Insect (Coleoptera)	Cry 4-genes DipIF (5' CAA GC CAAATCTTGTGGA-3') Cry 4-genesDipIR (5'ATGGCTTGTTTCGCTACATC 3')	933	Bacillus thuringiensis serovar coreanensis strain ST7.GenBank: CP016194.1
Км-2	Insect (Coleoptera)	Cry 4-genes DipIF (5' CAA GC CAAATCTTGTGGA-3') Cry 4-genes DipIR (5' ATGGCTTGT TTCGCTACATC-3')	745	Bacillus thuringiensis serovar kurstaki strain HD 1 GenBank: CP010005.1
2S	Soil	Cry 2-genes II (+) (5' - TAAAGAAAGTGGGGAGTCTT-3') Cry 2-genes II (-) (5' -AACTCCA TCGTTATTGTAG-3')	1421	Bacillus thuringiensis partial 16S rRNA gene, strain B20 GenBank: LN890196.1
3S	Soil	Cry 2-genes II (+) (5' - TAAAGAAAGTGGGGAGTCTT-3') Cry 2-genes II (-) (5' -AACTCCAT CGTTATTGTAG-3')	1500	Bacillus thuringiensis strain Gaoshi-1 16S ribosomal GenBank : GU201858.1
К-3	Insect (Coleoptera)	Cry 2-genes II (+) (5' - TAAAGAAAGTGGGGAGTCTT-3') Cry 2-genes II (-) (5' - AACTCCATCGTTATTGTAG-3')	1478	Bacillus thuringiensis serovar kurstaki strain NCIM 2 GenBank: KR109266.1
КМ-1	Insect (Lepidoptera)	Cry 3 - genes Coll F (5' - GTCCGTATATTCAGGTG-3') Cry 3 - genes Coll R (5' - CACTTAATCCTGTGACGCCT-3'	933	Bacillus thuringiensis serovar Galleriae strain HD-29, Complete genome GenBank: CP010089.1
Б-1	Soil	Cry 3 - genes Coll F (5' - GTCCGTATATTCAGGTG-3') Cry 3 - genes Coll R (5' - CACTTAATCCTGTGACGCCT-3')	933	Bacillus thuringiensis serovar Galleriae strain HD-29, Complete genome GenBank: CP010089.1
KS	Soil	Lep 2F (5' -CCGAAAGTCAA ACATGCG-3') Lep 2R (5' -TACATGCCCTTTCA CGTTCC-3')	933	Bacillus sp. B25(2016b) genome GenBank: CP016285.1

DOI:10.26104/IVK.2019.45.557

Conclusion

Fifty one samples from different habitats in North Kyrgyzstan`s districts were collected. 30 colonies were isolated based on the colony morphology of Bt were selected. After observation by phase-contrast microscopy Bt isolates were found in 15 samples out of the 51 samples analysed. The highest percentage of samples containing the bacterium was in soils. Biochemical tests have relieved the amylase, catalase, proteolysis and hydrolysis activities of obtained BT isolates. The results of PCR analysis have revealed natural isolates from soils, that were identified as *Bacillus thuringiensis* serovar galleriae with *Cry3* genes, *Bacillus thuringiensis* partial 16S rRNA gene and *Bacillus thuringiensis* strain Gaoshi-1 16S with *Cry2* genes, also *Bacillus* sp. B25(2016b) genome with *Lep2* genes. Isolated from insect bodies natural isolates were identified as *Bacillus thuringiensis* serovar coreanensis with Cry 4 genes and *Bacillus thuringiensis* serovar kurstaki with *Cry4* and *Cry2*-genes, also *Bacillus thuringiensis* serovar galleriae with *Cry3* - genes.

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