

# Biodiversity of *Bacillus thuringiensis* Strains and Their *Cry* Genes in Ecosystems of Kyrgyzstan

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## Abstract

The present study aims to isolate the unknown and known serotypes of *Bacillus thuringiensis* (Bt) from natural objects in Kyrgyzstan. A total of 83 Bt strains were isolated from natural substrates, of which 30% were taken from the soil and litter samples, 69.7% from dead insects and about 0.3% from slugs. Serological examination revealed that such subspecies as *var. thuringiensis* (H-1), *var. alesti* (H-3), *var. sotto* (H-4a4b) and *var. entomocidus* (H-6) predominated in the upper horizon of soils in all climatic zones. In the dead insects such species as *subsp. thuringiensis*, *subsp. galleria*, *subsp. sotto*, *subsp. kurstaki*, *subsp. aizawai* and *subsp. entomocidus* dominated. A set of Bt strains isolated from insects and soil samples, selected from different ecosystems in Kyrgyzstan was molecular taxonomically characterized using the *pycA* gene as marker for phylogenetic reconstruction. Within the *Bacillus cereus sensu lato* species complex, all Kyrgyz isolates were shown to belong to the *B. cereus subspecies thuringiensis*. Most isolates were assigned to the lineage *Bt tolworthi*, with two isolates each belonging to the lineages *Bt kurstaki* and *Bt sotto*. A high degree of *cry* gene diversity was demonstrated in the set of Bt isolates, with several gene copies simultaneously present in a single strain; a particularly conspicuous trait was the frequent combination of Lepidopteran-specific *cryI* with Dipteran-specific *cryIV* genes in the same Bt isolate.

## Keywords

*Bacillus thuringiensis* Serotypes in Nature, 16S rRNA Gene Diversity of Bt Isolates, *Cry* Gene Diversity

## 1. Introduction

*Bacillus thuringiensis* is a gram-positive, spore-forming bacterium with a wide

insecticidal activity against numerous organisms such as mites, nematodes, protozoans, and insects [1]. The Cry proteins or delta endotoxins produced by this bacterium are the most well-known insecticidal proteins and have been extensively used for biocontrol of pests from the orders Diptera, Coleoptera, Homoptera, Lepidoptera, Hymenoptera, Orthoptera [2]-[8]. The optimal condition for the Cry toxin to grow and sporulate is in the insect's alkaline gut. This facilitates the ability to infect insect guts. The primary action of Cry proteins is to lyse midgut epithelial cells through insertion into the target membrane and form pores [9]. Once ingested, crystals are solubilized in the alkaline environment of midgut lumen and activated by host proteases [10].

The family of genes coding for these toxins is the *cry* gene family and at the present about 304 such cry holotype protein genes have been reported [11]. These insecticidal crystal protein genes are the major source for the development of insect-resistant transgenic plants [12] [13]. Genetically engineered crops which produce insecticidal proteins of Bt for the control of pests have been planted on a cumulative total of 560 million hectares worldwide since 1996 [14]. Due to extensive use of transgenic crops in developing countries based on *cry*-type genes, there is a need for alternative *cry* gene sequences to meet the challenge of novel insect resistance [15] [16] [17] [18]. Crucial to this development is the identification of novel and more active strains with respect to insect pests of economically important crops [19].

Meadows [20] has analyzed three prevailing hypothetical niches of *B. thuringiensis* in the environment: as an entomopathogen, as a phylloplane inhabitant, and as a soil microorganism.

Due to the reduction and pollution of natural habitats of these bacteria, there is a threat of overloading and recharging of ecological niches by existing subspecies of *B. thuringiensis*. Pristine habitats are an important source of new strains with new insecticidal genes and still intact ecosystems are becoming on the earth even less and less every year. Study pristine and still untouched ecological niches as sources to obtain new isolates with a unique combination of genes will open the extensive possibility of bacterial agents with novel properties.

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 [21]. Consequently, before we began to isolate new isolates of Bt we had two objectives: first, to search for already known and in use serotypes, and secondly, to search for new subspecies able to show specific biological activities to different pest species. When we selected a known serotype, it was necessary to follow three basic principles. First, each serotype may be isolated from one insect species or from other substrate. Second, each serotype may be isolated from several insect species and other substrate.

Third, several serotypes of BT may be isolated from one insect species or from other substrates. A different approach was used to search for new serotypes that will be active against certain pest species.

To isolate the unknown and known serotypes, and to define their distribution in nature in Kyrgyzstan, the materials for research were collected mainly in the landscapes less subjected to anthropogenic influences.

## **2. Methods and Material**

### **2.1. Natural Sources for the Isolation of BT Strains**

Soil, plant litter, plants and sick and dead insect samples were collected in natural landscapes of different regions in Kyrgyzstan as sources for the isolation of Bt species.

#### **2.1.1. Isolation of BT Strains from Soil**

We attempted to collect soil from locations that were as diverse as possible. Soil samples were collected by scraping off surface material with a sterile spatula and then obtaining approximately 100 g samples from 2 - 10 cm below the surface. Samples were then stored at 4°C until use. To provide the isolation of Bt species from soil, soil samples were analyzed using the acetate selection [22] and the methods of soil microbiology and biochemistry [23] with some modifications. 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 subcultured on new NA plates until pure cultures were obtained, and they were kept at 4°C for further identification.

#### **2.1.2. Isolation of BT Strains from Insect Samples**

The surface of the insect's body was previously sterilized in the 70° hydrated alcohols for 2 - 3 min to avoid contact with foreign microflora, then was flamed in a spirit 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°C - 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 meat-peptone agar and incubated at 27°C - 28°C.

#### **2.1.3. Isolation of BT Strains from Plant Samples**

Shredded plant litter and plant substrates were pre-heated in the oven at 80°C for 30 min to remove Gram-negative bacteria and immersed in sterile saline solution (NaCl, 0.85%) and repeatedly washed. To 10 mL of Luria-Bertani broth, 1

g from each sample was added and buffered with sodium acetate (0.25 M, pH 6.8) in a 125 mL flask. The dilutions  $10^{-1}$ ,  $10^{-4}$  and  $10^{-6}$  of each sample were prepared and planted on medium from  $10^{-4}$  to  $10^{-6}$  dilutions. A medium composition (g/:a) peptone-2; glucose-1; yeast extract-1; casein hydrolysed-1; agar-2; the tap water. b) soluble starch-10; casein-1;  $\text{NA}_2\text{HPO}_4$ -0,5; yeast extract-1; agar-2; the water tap was used for the isolation of BT strains from plant litter and plant substrates.

#### **2.1.4. Stain Methods for the Detection of Crystal Bodies and Spores**

The three-colour stain method [24] was used for the detection of crystals and spores. Saturated solution (5.0%) of malachite green was spread at a prepared uniform smear from bacterial cultures and heated on the flame of a spirit lamp to the point of the intense discharge of vapours. Glass slides were cooled and gently washed with distilled water. Acetic acetone solution of amid black 10 B was flooded at the wet glass surface for one minute. Excess dye was poured off and washed gently in tap water and the slides were drained against a paper towel. The smear was then stained with 0.5% solution of neutral red for 30 - 40 seconds, washed and dried. As the result of staining, spores were coloured in emerald green with red rims. The crystals had a blue-violet or black colour. Vegetative cells were red-brown or pink. Frequently, we used a simple staining technique with 5.0% aqueous solution of carbolic eosin. This solution was spread on the surface of the smear and heated on the flame until the point of an intense discharge of fumes, stained for 2 min and washed in tap water and drained. Stained glass slides were examined under an oil immersion microscope (Leica DM2500 P) at 100X resolution. As the result of colour staining, the crystals were coloured in red; spores were not stained with a red border, and vegetative cells were red stained.

#### **2.1.5. Phenotypic Characterization of BT Strains**

The isolated bacterial cultures were studied for their ability to grow on meat-peptone broth, meat-peptone agar and sliced potatoes; to produce a pigment; in formation of cells in the liquid substrates; the nature of sediment; turbidity and smell. 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 ( $\beta$ -glucosidase, esculin), hydrolysis (protease, gelatin), hydrolysis  $\beta$ -galactosidase (para-Nitrophenyl- $\beta$ -D-galactopyranosidase) and 12 assimilation tests, with substrates such as glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, 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 [25] [26] [27].

#### **2.1.6. H Serotyping**

The H antigen structure of local Bt strains was studied by flagellar agglutination

reaction. Standard classic strains and antiserum to below Bt serotypes, including *subsp. thuringiensis* (H-1), *subsp. finitimus* (H-2), *subsp. alesti* (H-3a 3c), *subsp. kurstaki* (H-3a 3b3c), *subsp. kenia* (H-4a4c), *subsp. sotto* (H-4a 4b), *subsp. gal-leria* (H-5a 5b), *subsp. entomocidus* (H-6), *subsp. aizawae* (H-7) and *subsp. morrisoni* (H-8a 8b) were received from the Pasteur Institute in Paris. The sero-type was determined by tube agglutination tests using diluted antisera, as de-scribed by [28]. Tests were repeated twice to confirm the results. Intraspecific differences of Bt were determined by the circuit-key of H.d Barjac, A. Bonnefoi [29] and by other manuals [30].

### 2.1.7. PCR Analysis

Purified bacteria were incubated in MPB for 24 h at 28°C. Cells harvested at the early exponential growth phase were transferred to Eppendorf tubes containing lysis buffer (50 mM Tris, 50 mM EDTA, 3% SDS, 1% BME, pH 7.2) and heated at 65°C for 20 min with occasional gentle mixing. The lysate was extracted with equal volumes of phenol, chloroform, isoamyl alcohol (25:24:1) and phenol (wa-ter saturated) once. The aqueous phase was removed after centrifugation (15,000 rpm, 15 min, and 4°C). The total DNA was precipitated by isopropanol and pel-let washed with 70% ethanol. Finally, pellets were air dried and suspended in 200 µl TE buffer (0.1 mM Tris HCL, 1 mM EDTA pH 8.0) and allocates were directly utilized for PCR assay. Amplification was performed with a Multigene Thermal Cycler (TC9600-G/TC, Labnet International), by using a 25 µl mixture contain-ing 15 µl of PCR Master Mix (*Taq* DNA polymerase, MgCl<sub>2</sub>, deoxyribonucleo-tide triphosphate and reaction buffer), 2 µl of each primer, 1 µl of template DNA and 1 µl of H<sub>2</sub>O. The amplification program was used as follows: 95°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 1, 5 min. PCR products were electrophoresed in a 1.0% agarose gel and visualized by BioDoc-It™ Imag-ing Systems (Ultra-Violet Products Ltd.) after ethidium bromide staining. To control the contamination, there was a negative control reaction, in that sterile water was added as a matrix.

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.* [31]. Screening for *cry* genes was carried out using the fami-ly-specific primer pairs directed toward the identification of some of the main groups of *cry* genes.

Sequence analysis was performed by the MacroGen Company (10F World Meridian Center, #60-24 Gasan-dong, Geumchun-gu Seoul, Korea, 153-023) and sequences were edited with Applied Biosystems 3730XL sequencers. Only se-quences with >700 nucleotides were used for diversity analyses. The phylogenet-ic relatedness among different sites was determined using the Cluster environ-ment. The 16S rRNA gene sequences were deposited in the Gen Bank and DB of

NCBI nucleotide sequence databases. Sequences were compared with the NCBI public nucleotide database at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> using BLAST (blast). The sequences were aligned Using Cluster W and checked manually using the software MEGA4 [32].

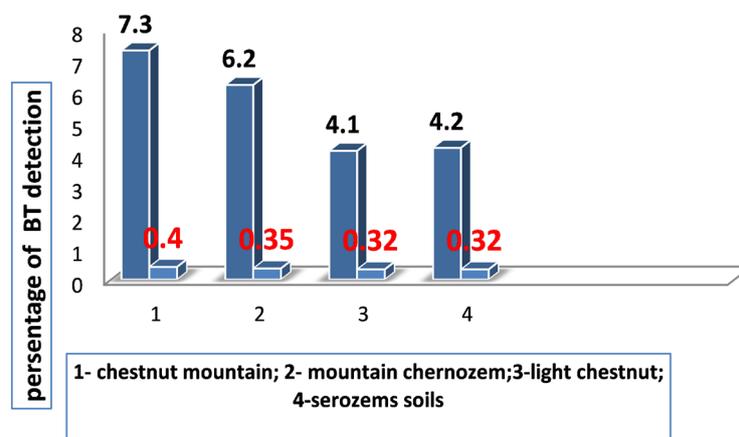
### 3. Results

#### 3.1. Isolation of Bt Strains from Natural Objects in Kyrgyzstan

In total, 240 soil samples, 64 litter samples, 786 insect bodies and 23 slugs were collected from different ecosystems of Kyrgyzstan during 2014-2016. A total of 83 Bt strains were isolated from these natural substrates, of which 30% were from the soil and litter samples, 69.7% were from the body of the dead insects, about 0.3% were from slugs.

**Table 1** has shown that, in general, the frequency of the crystal-forming bacteria in soils of Kyrgyzstan was rather significant (25.7%, and 4.3% from litter samples). Bt species were obtained from the litter in the association of cellulolytic ammonifiers. In particular, mountain chestnut soils, mountain chernozem, light chestnut and grey soils were significantly rich with Bt species. Spore-forming bacteria in these soils types comprised 4.2% - 7.3%, of which the crystal-forming bacteria have a share of 0.3% - 0.4% (**Figure 1**).

A wide variety of insects from different taxonomic groups, whose dead bodies were found in the steppes, foothills, mountains and pastoral areas, were examined in this study. A high incidence of crystal-forming bacteria was found in *Lepidoptera*, in particular in cotton bollworm and cabbage moth bodies, such that the detection percentage of Bt reached 20.0% - 20.75%. The beetles (*Coleoptera*) were also potential carriers of Bt strains. Consequently, a significant number of Bt strains were isolated from dead dung beetles and other beetles (17.2% - 18.5%), while the crystal-forming bacteria were not obtained from other coleopteran insects like the cotton weevil, rice weevil and ladybird. From the dead bodies of Italian locusts (*Orthoptera*) a small number of Bt strains were



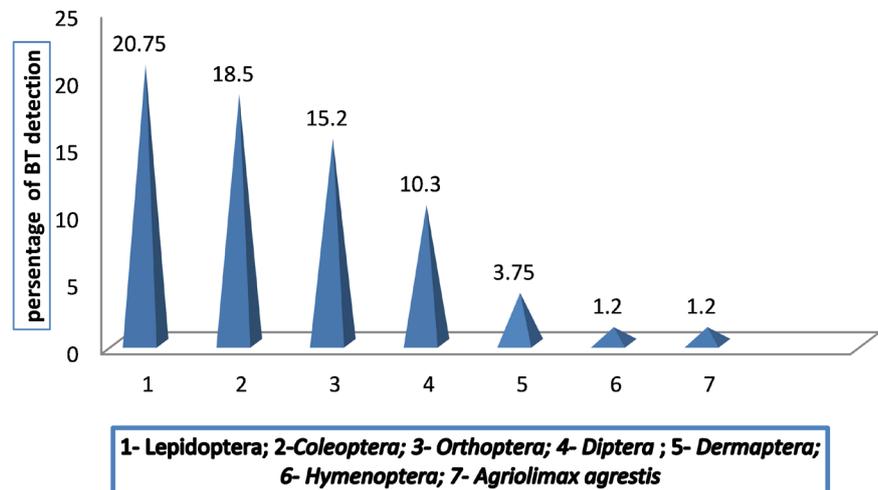
**Figure 1.** The detection frequency of the crystal-forming bacteria in different types of soils.

**Table 1.** Distribution of crystal-forming bacteria in natural objects of Kyrgyzstan.

№	The test object	Number of examined objects, specimens	Number of selected of <i>Bt.</i> isolates	Percentage of occurrence of <i>Bt.</i> isolates
1	Soil	240	84	35.0 ± 0.57
2	The litter	62	9	14.5 ± 0.32
3.	<b>The bodies of dead insects in nature</b>			
	<b><i>Order Lepidoptera</i></b>			
3.1	• Cotton bollworm caterpillar ( <i>Helicoverpa armigera</i> Hh)	82	17	20.7 ± 0.65
	• Cutworm caterpillar ( <i>Scotia seedtime</i> Schiff)	47	9	19.1 ± 0.53
	• Cabbage moth caterpillar ( <i>Luella maculipennis</i> Curt)	53	11	20.75 ± 0.63
	• Gypsy moth caterpillar ( <i>Lymintaria dispar</i> L)	100	20	20.0 ± 0.48
	• Sea buckthorn moth caterpillar ( <i>Gelechia hippophaella</i> )	50	5	10.0 ± 0.13
		<b>332</b>		
3.2	<b>Order Coleoptera</b>			
	• Dung beetle ( <i>Capris minutes</i> )	120	22	18.5 ± 0.41
	• Beetle rape ( <i>Entomoscelis adonicis</i> Pall)	47	8	17.0 ± 0.27
	• Darkling beetles arthropods	43	7.7	18.0 ± 0.25
		<b>210</b>		
3.3.	<b><i>Order Orthoptera</i></b>			
	• Italian locust ( <i>Calliptamus italicus</i> L.)	75	10	13.7 ± 0.17
	• Brown horse ( <i>Chorthippus apricarius</i> )	63	9.0	15.2 ± 0.21
		<b>138</b>		
3.4	<b><i>Order Diptera</i></b>			
	• Tephritid ( <i>Tephritidae</i> )	28	3	10.7 ± 0.12
	• Onion fly ( <i>Delia antique</i> Mg.)	24	2	7.14 ± 0.14
		<b>52</b>		
3.5	<b><i>Order Dermaptera</i> Earwigs (<i>Forficula auricularia</i>)</b>	22	1	3.75 ± 0.15
3.6	<b><i>Order Hymenoptera</i> Beer (<i>Sceliph</i>)</b>	32	1	1.2 ± 0.04
3.7	<b><i>Type off Mollusca—Slugs (Agriolimax agrestis)</i></b>	23	12	1.2 ± 0.03

isolated, whereas a higher density of these bacteria have been found (13.7% - 15.2%) in the bodies of the brown horse (*Orthoptera*). About 10.3% *Bt* strains were isolated from *Diptera* insects, and the *Dermaptera* and *Hymenoptera* orders contained a small amount of *Bt* spores and cells (3.75% and 1.2%, respectively). Colonies were isolated from the bodies of 32 bees (*Sceliph*). It was particularly interesting that some *Bt* isolates (1.2%) were obtained from slugs (*Agriolimax agrestis*) (Figure 2).

The results of these studies suggest that these bacteria have some redistribution in the investigated ecological niches. Such subspecies as *var. thuringiensis*



**Figure 2.** The detection percentage of Bt strains from dead insects of different orders and from slugs.

(H-1), *var. alesti* (H-3), *var. sotto* (H-4a4b) and *var. entomocidus* (H-6) predominated in the upper horizon of soils in all climatic zones. The greatest number of these bacteria was found in brown and grey, and smaller numbers were found in the mountain forest soils (Table 1).

A high prevalence of crystal forming bacteria in the cadavers of butterflies, beetles and *Orthopterans* insects was discovered. However, the spores were found in their hosts only in very small quantities (only one colony per insect). Therefore, we should assume that in nature there are often more enzootic seats of *Bt* than epizootic. These obtained results confirm the opinion of some researchers that *Bt* is not primarily a saprophyte and does not require the assistance of commensal bacteria but is a true pathogen in its own right and furthermore that its primary means of reproduction is in an insect cadaver [33]. Other scientists note that *B. thuringiensis* spores could germinate and multiply in particular conditions in the insect larvae, which allows the spreading in surrounding areas [34].

The ecological role of *B. thuringiensis* in the soil ecosystem is poorly understood. According to some data soil is a potential source for Bt strains. *Bacillus thuringiensis*-like strains were isolated from 95 of 413 samples collected at the 0 - 5 cm depth of no cultivated soils and stagnant or dried-up ponds as well as from dust from stored grain products in South Central United States. The most frequently isolated strains were *kurstaki*, *aizawai*, *morrisoni*, *thuringiensis*, *sotto* and *kenyae* that together represented more than 90% of the characterized isolates [35].

The wide spread of some serotypes of Bt in soil can be explained [30] by an ability to produce bacteriocins or other antibiotic agents, which allow them to compete and survive in soil habitats.

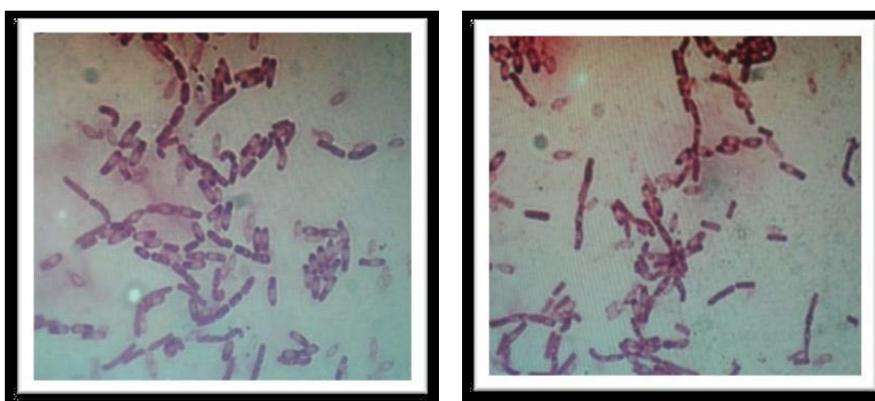
Thus, soil, plants, diseased and dead insects could serve as the natural reservoirs and keepers of the entomopathogenic bacteria Bt in nature.

### 3.2. Phenotypic/Physiological and Biochemical Characterization of Bt Isolates from Kyrgyzstan

The phenotypic characterization tests have indicated that natural isolated were Gram-positive, spore and crystal forming, motile and catalase was positive. Indole was not produced. Nitrate was reduced and only some strains were not able to reduce it. Acid was not produced from glucose. Oxidase was variable. Trisodium citrate was assimilated except in four strains. Esculin ferric citrate was hydrolysed by  $\beta$ -glucosidase. Carbon substrate assimilation was variable between strains. These strains could be assigned to the genus *Bacillus*.

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 hydrolyze gelatin, esculin ferric citrate and starch. The ability to hydrolyze cellobiose, lecithin and urea was variable. Among the obtained natural strains some of them were able to form films on the MPB, to produce an exotoxin. Pigment production was unstable with a short expression time. Several serovars give the same reactions (such as *kurstaki*, *kenyae*, *aizawai*) or differed only in some variable characteristics (such as cellobiose fermentation for *sotto* and *dendrolimus*). These local strains differed in the time of sporulation. Sporulation began in most strains after 18 - 24 hours, and in the others after 36 hours of incubation. Sporulation was completed after 48 h, when most of the Bt cells contained spores and protein crystals. The endospores in all the Bt isolates were either paracentral or sub-terminal (**Figure 3**). The strains had different configurations of crystals; including diamond shaped, square, lemon shaped, round or shapeless. Most strains produced crystals of different shapes; some strains have only one type of crystal.

Differentiating physiological and biochemical characteristics of natural isolates of Bt isolated from ecosystems of Kyrgyzstan have been shown in **Table 2**.



**Figure 3.** The sporulation phase in most Bt strains after 18 - 24 hours of incubation, staining with 5.0% aqueous solution of carbolic eosin, under an oil immersion microscope (Leica DM2500 P) at 100X resolution.

**Table 2.** Phenotypic characterization of natural isolates of *Bacillus thuringiensis*.

Phenotypical characterization	<i>Subsp. kurstaki</i>	<i>Subsp. aizawai</i>	<i>Subsp. morrisoni</i>	<i>Subsp. kenya</i>	<i>Subsp. sotto</i>	<i>Subsp. thuringiensis</i>	<i>Subsp. galleria</i>	<i>Subsp. entomosisidis</i>
Voges Proskauer reaction	+	+	+	+	+	+	+	-
Fermentation of sucrose with an acid	-	-	+	-	+	-	-	+
-of mannose	-	-	-	-	-	-	-	+
-of cellobiose	+	+	+	+	-	+	+	+
Hydrolysis -of salicine	+	+	-	+	-	+	+	+
-of esculin	+	+	+	+	+	+	+	+
-of starch	+	+	±	±	++	±	+	+
-of lecithin	+	+	-	+	+	+	-	-
-of urea	+	+	-	+	-	+	-	-
-of gelatin	+	+	+	+	++	-	+	+
Pigment production	-	-	-	-	-	-	-	-
Films on the MPB (meat-peptone broth)	-	-	+	-	-	-	+	-
exotoxin production	-	-	+	±	-	+	-	-

Note: +- presence of characteristic; - a BT absence of characteristic; ±- variability of characteristic.

### 3.3. Serological Characterization of BT Isolates from Kyrgyzstan

The hag gene encodes flagellin, which is responsible for eliciting the immunological reaction in H serotyping. Specific flagellin amino acid sequences have been correlated to specific Bt H serotypes and at least 69 H serotypes and 82 serological varieties (serovars) of Bt have been characterized [28]. H serotyping, however, is limited in its capability to distinguish strains from the same H serotype or from the same serovar [36].

The differential key designed to determine the biochemical and phenotypic characteristics of Bt strains provides a basis for categorizing newly obtained natural isolates into a Bt group, but to fully explicate their differences at the subspecies level, it is necessary to explore an H antigen of flagellar protein units of available strains. Other scientists argue that most serovars cannot be distinguished by biochemical characters. H serotyping still remains the simplest specific way to classify Bt strains. Serovars are well-established entities characterized by specific H antigens, and which enable comparisons to be made between the data of various researchers [26].

The H antigen structure of local Bt strains was studied with a flagellar agglutination reaction. This revealed 54 strains that were found to comprise eight serotypes, by means of agglutination with standard antiserum and by comparison to standard Bt. Of these, 15 natural strains were identified as *subsp. thuringiensis* (H-1), 12 strains as *subsp. kurstaki* (H-3a 3b3c), nine strains as *subsp. galleria*

(H-5a 5b), seven strains as *subsp. sotto* (H-4a 4b), five strains as *subsp. aizawai* (H-7), three strains as *subsp. entomocidus* (H-6), two strains as *subsp. kenia* (H-4a4c) and one strain as *subsp. morrisoni* (H-8a 8b).

When analyzing the detected locations of Bt strains, it was revealed they are confined to certain ecological niches. In natural biogeocenoses of Central Tien-Shan and Issyk-Kul district such species as *subsp. thuringiensis*, *subsp. gal-leria*, *subsp. sotto*, *subsp. kurstaki* were dominant in the dead bodies of insects, whereas in the ecosystems of the Inner Tien-Shan and Alay districts *subsp. ai-zawai*, *subsp. entomocidus* and *subsp. kurstaki* were most prevalent (**Table 3**).

### 3.4. Molecular Characterization of Bt Strains Isolated from Ecosystems of Kyrgyzstan

Besides the phenotypic, biochemical and serological characterization, the taxonomic identity of the crystalliferous isolates was confirmed by amplification

**Table 3.** The distribution of crystal forming bacteria in the ecosystems of Kyrgyzstan.

Ecosystems	Dominant serotypes							
	H-1	H-3a3b	H-4a 4b	H-5a 5b	H-6	H-7	H-8	H-4a 4c
<b>1) Issyk-Kul and the Central Tien Shan biogeographically districts:</b>								
- fir forests	+	+			+			
- mid forests	+	+	+	+	+		+	
- mid-steppes	+	+	+	+			+	
- rain fed northern areas	+	+	+		+		+	
- lakes and wetlands	+				+			
<b>2) Internal Tien-Shan biogeographically district:</b>								
- juniper forests	+		+	+				+
- tugai forests	+			+		+		
- cryophyte meadows	+					+		
- cryophyte steppes		+	+			+	+	
- cryophyte desert	+	+	+					
<b>3) West-Tien-Shan, Fergana and Alai biogeographically districts:</b>								
- fir forests	+	+				+		
- juniper forests				+	+			
- broadleaf forests	+	+						
- almond and pistachio			+		+	+		
- middle mountain meadows				+	+			
- foothill steppes		+		+	+			
- foothill deserts	+	+		+	+			
- lakes and wetlands	+							

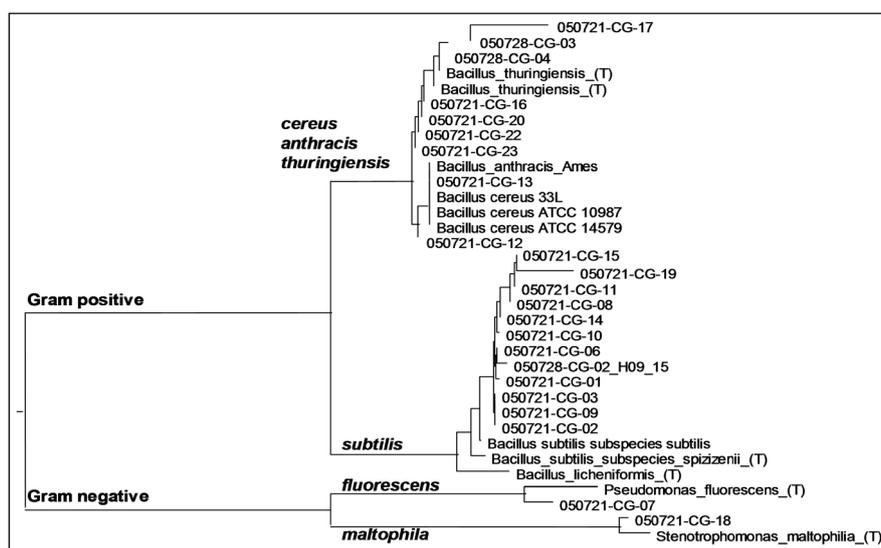
and partial sequencing of their 16S rDNA genes. In this process, 40 Bt strains isolated from soil sources and the bodies of insects selected in different ecological niches were analyzed for a partial sequence (700 - 800 bp) of the 16S rRNA encoding gene. The partial 16S rDNA sequences were tested by BLAST analysis against the GenBank data base (Table 4). Comparison of sequence data among isolates and with standard *Bacillus* strains (Figure 4) has confirmed assessment of the taxonomic genus *Bacillus* for all isolates. Within the genus, some isolates were demonstrated to belong to the *B. cereus* group that together several *B. cereus* subspecies comprises both *B. thuringiensis* and *B. anthracis*. This confirms the view of other scientists that Bt is a variety of *B. cereus* along with *B. anthracis* and *B. mycooides* [37] and certainly Bt can share many common phenotypic and genotypic properties, to the extent that the three species have been placed under one group called the *Bacillus cereus* (BC) group. Together with the human pathogens *Bacillus cereus* (*B. cereus sensu stricto*) and *B. anthracis*, the insect pathogen *B. thuringiensis* belongs to the *Bacillus cereus* group (*B. cereus sensu lato*) that—on the basis of 16S rRNA gene sequence comparisons—should be regarded as one single species.

When screening these natural Bt isolates on test insects, 13 strains have showed a significant entomopathogenic activity to larvae stage of Lepidoptera pests. They have a variety of biological and geographical origin and represent the southern and northern parts of the country located at different altitudes (Table 5).

Phylogenetic reconstruction from *pycA* gene sequences (Figure 5) firmly, *i.e.* with 99 % bootstrap support, assigns these isolates to the subspecies *B. thuringiensis* within the *B. cereus sensu lato* complex.

**Table 4.** Primer sequences used for amplification of 16S rDNA genes.

Kyrgyz Strain Designation	Primer pairs	Positions	Gene(s) recognized	Product size (bp)	Nucleotide sequence
24-4a;24-4b;24-4c; 24-4d;24 -4e	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.
55-T; 55-Ta, 55-Tb; 55-Tc; 55-Td	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.
12-06; 12-07;12-08; 12-09	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.
8-sov; 8-sov-1;8-sov-2; 8-sov-3; 8-sov-4; 8-sov-5	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.
60-H-3; 60-H-4; 60-H-5; 60-H-7;	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.
23-2; 23-3; 23-4; 23-5; 23-6; 23-7.	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.
66-1; 66-2; 66-3; 66-4; 66-5;	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	
62- 1; 62-2; 62-3; 62-4; 62-5	27F-HT and 1492R-HT	1472 - 2029	16SrRNA	700 - 800	5.-AGAGTTTGATCCTGGCTCAG-3. 5.-GGTTACCTTGTTACGACTT-3.

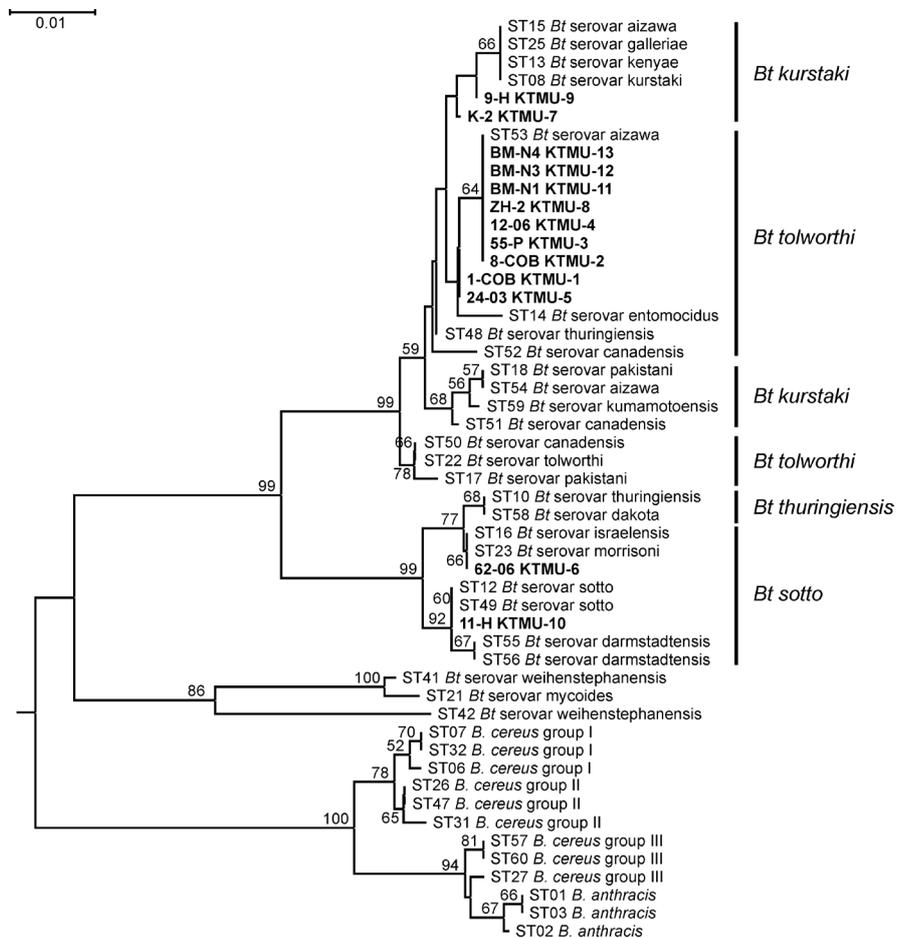


**Figure 4.** Neighbour-joining phylogenetic tree based on 16SrRNA gene sequences showing the position of isolated Bt strains from environmental sites. Numbers at branching points represent bootstrap values from 100 replicates. The size bar corresponds to 5% nucleotide sequence divergence.

**Table 5.** *B. thuringiensis* isolates with significant entomopathogenic activity (NK and SK designate the Northern and Southern part of Kyrgyzstan, respectively).

Strain	Geographic Origen	Isolation Source
KTMU 1	Naryn region (NK)	Locust <i>Calliptamus turanicus</i> (Serg. Tarb)
KTMU 2	Osh region (SK)	Turnip moth <i>Scotia segetum</i> (Schiff)
KTMU 3	Batken region (SK)	Soil
KTMU 4	Naryn region (NK)	Leaf beetle <i>Lygus pratensis</i> (L.)
KTMU 5	Batken region (SK)	Soil
KTMU 6	Walnut forest Arslanbob (SK)	Litter
KTMU 7	Chy region (NK)	Cockchafer <i>Melolontha melolontha</i> (L.)
KTMU 8	Chy region (NK)	May Beetles (June Bugs), <i>Phyllophaga</i> spp.
KTMU 9	Walnut forest Arslanbob (SK)	Gipsy-moth <i>Ocneria dispar</i> (L.)
KTMU 10	Walnut forest Arslanbob (SK)	Gipsy-moth <i>Ocneria dispar</i> (L.)
KTMU 11	Chy region (NK)	Wax moth larva <i>Galleria mellonella</i> (L.)
KTMU 12	Chy region (NK)	Wax moth larva <i>Galleria mellonella</i> (L.)
KTMU 13	Chy region (NK)	Wax moth larva <i>Galleria mellonella</i> (L.)

Sequence types of the subspecies *B. thuringiensis* are further subdivided into two clusters, the *Bt kurstaki/tolworthi* group and the *Bt thuringiensis/sotto* group; both the respective clades receive 99% bootstrap support in the *pycA* phylogeny. The investigated isolates from Kyrgyzstan are found unevenly distributed over both clades, with nine strains being provisionally assigned to the *Bt tolworthi* lineage and two each to *Bt kurstaki* and *Bt sotto*. Determination of further MLSA marker sequences for these isolates is currently under way.



**Figure 5.** Neighbour Joining (NJ) phylogeny of the *Bacillus cereus sensu lato* complex as reconstructed from *pycA* gene sequences. Terminal branches are labelled by sequence type (ST) numbers according to Jolley & Maiden [37] and the taxonomic assignment of the respective bacterial strain(s). Clustering into *B. thuringiensis* lineages is indicated at the right margin. Numbers on branches designate bootstrap support percentage values (>50%). The size bar corresponds to 5% nucleotide sequence divergence. The *pycA* gene of *Bacillus subtilis* has served as outgroup to root the tree.

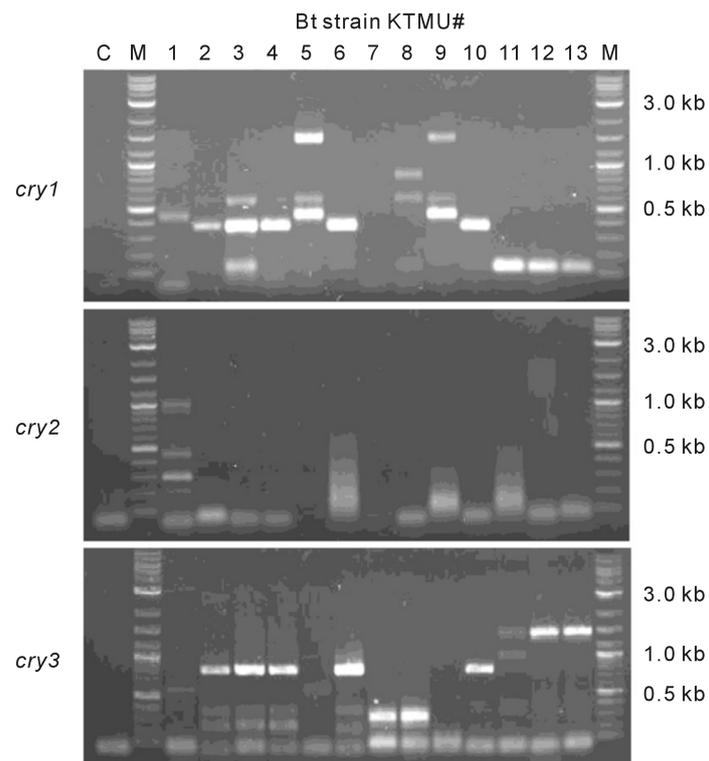
As geographic origins and source materials of the isolates are rather diverse (Table 5), a correlation of taxonomic assignments can hardly be obvious from the data. A notable exception are strains KTMU #11-13 stemming from the same region and host insect that are consistently assigned to the same lineage (*Bt tolworthi*); the same holds for isolates KTMU #3 and 5. However, isolates KTMU #9 and 10 that share both geographic origin and host have been assigned to the clearly distinct lineages *Bt kurstaki* and *Bt sotto*, a finding consistent with an elevated degree of local biodiversity.

### 3.5. 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 harbored on large plasmids where they often occur in clusters of different *cry* variants. A sin-

gle Bt strain can harbour numerous different insecticidal crystal protein genes from 46 known classes or primary ranks. The *cry1* primary rank is the best known and contains the highest number of *cry* genes which currently totals over 130 [38]. 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 Dipteran insects, respectively [11].

Targeted amplification of three different types of plasmid-encoded *cry* genes (Figure 6) demonstrates that all strains contain respective *cry* gene copies, with a pronounced bias in *cry* gene frequencies: whereas *cry1* or *cry4* genes are present in most strains, and often appear to be so in several—not necessarily functional or active—copies, according to PCR product numbers, *cry3* specific primers reproducibly gave positive signals only from isolate KTMU #1. A striking feature is the frequent combination of several *cry* gene types in a single isolate: only three out of 14 strains (KTMU #5, 7, and 9) contain *cry* genes belonging to a single type. This rather regular presence of several *cry* gene copies, potentially combining protein toxins of different specificities, within a single strain is of high interest with respect to the possible application of these strains for biocontrol purposes.



**Figure 6.** Agarose gel electrophoresis of PCR reactions using *cry1*, *cry3*, and *cry4* gene specific primer pairs (as indicated at the left-hand margin) with genomic DNA from *B. thuringiensis* isolates. Lanes are labelled by the isolate numbers given in Table 1; lane labels “C” and “M” designate negative controls and DNA size standards, respectively. DNA molecule sizes are indicated at the right margin.

The assessed distribution of *cry* genes does not reveal an obvious correlation with taxonomic assignments, geographic origin or source material with once more the notable exception of strains KTMU #11-13. However, the three pairs of isolates from the same region and same or similar hosts, *i.e.* KTMU #3 and 5, #7 and 8 as well as #9 and 10, display pairs of highly diverse *cry* gene amplification patterns.

#### 4. Discussion

As results have shown *B. thuringiensis* seems to be indigenous to many environments, its strains have been isolated from many habitats, including soil, litter, insect bodies and slugs, collected from different ecosystems of Kyrgyzstan, where no *Bt* products have been applied before. Although *Bt* was found in all the samples, its occurrence in the insect bodies was found to be relatively high compared to other types of samples. The existence of the enzootic foci of entomopathogenic bacteria like *Bt* and micro-distribution such centers have found in high meadows, middle mountain steppes, deserts, coniferous and walnut forests. In spite of the difficult terrain, scale heights (from 1500 to 3000 m) and the severity of climatic conditions, *Bt* entomopathogenic bacteria as a biological species function in these ecological niches. Other researchers have also noted that *Bt* is subject to wide variations in temperature, humidity, nutrient and oxygen availability; and probably occurs within consortia or in direct competition with other micro- and macroorganisms [39]. The obtained data allow assuming that, unlike in laboratory conditions the growth or sporulation of *Bacillus* spp. in their natural habitats are certainly slower, probably these bacteria can form microcolonies on and within soil aggregates, plant debris and corpses of insects. Apparently, their parasitic essence can show itself in specific and favourable conditions, and then they can become the pathogens of certain insects. This possibility can be set up with an increase of pest population density at the time of its mass outbreak. Consequently, the presence of these bacteria in the intestinal microbial association of insects provides them with wide dissemination. Their function and life forms in different ecological niches can show differently. We also suggest that in a certain concentration required for the disease, these bacteria are leading a parasitic life in the living insects, developing in their insides and causing their death, and can behave as obligate pathogens.

Isolated *Bt* strains were characterized by biochemical tests and serological reaction by exploring an H antigen of flagellar protein units of strains to definite the serotypes. When analyzing the detected locations of *Bt* strains, it was revealed they are confined to certain ecological niches. In natural biogeocenoses of the Central Tien-Shan and Issyk-Kul districts the insects were dominated by such species as *subsp. thuringiensis*, *subsp. galleria*, *subsp. sotto* and *subsp. kurstaki*, whereas in the ecosystems of the Inner Tien-Shan and Alay districts *subsp. aizawai*, *subsp. entomocidus* and *subsp. kustaki* were prevalent in the insects.

The taxonomic identity of the crystalliferous isolates was confirmed also by amplification and partial sequencing of their 16S rDNA genes and *pycA* gene. New subspecies of this bacterium have been discovered by molecular characterization, such as *Bacillus thuringiensis* var. *kurstaki*, var. *tolworthi* and var. *sot-to*, which usually are more characteristic for Asian regions.

Experiments revealed pronounced differences in the frequency of *cry* genes in Kyrgyz Bt isolates. Whereas genes encoding potentially lepidopteran-specific Cry1 and dipteran-specific Cry4 proteins were regularly detected, genes for coleopteran specific Cry3 toxins were found present in only a small number of the strains investigated. Moreover, *one or several cry1 genes frequently accompanied cry4 genes*.

The observed accumulation of *cry* genes expected to display different activities is of obvious interest from a biocontrol perspective.

Thus, the results have showed that the ecological niches not exposed to anthropogenic influences can serve as a rich source for isolation of such biological agent as *Bt* with a new combination of *cry* genes. Among obtained new strains are the best candidates for the development of new biological products with a different spectrum of activity against several species of insects.

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