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EXPERIMENTAL ARTICLES

Interspecies Relations between *Bacillus thuringiensis* Strains Studied by AP-PCR and Sequence Analysis of Ribosomal Operon Regions

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Abstract—The interspecies relationships between *Bacillus thuringiensis* strains producing different types of δ-endotoxins were studied using a range of molecular-biological methods. Analysis of the 16S rRNA nucleotide sequence, the 16S to 23S rRNA intergenic spacer sequence, and the 5'-terminal region of 23S rRNA allowed the studied strains to be subdivided into three groups based on the pattern of nucleotide substitutions. In terms of the pattern of substitutions, the strains of the first group are similar to the *B. thuringiensis* type strain ATCC 10792^T, the strains of the second group are practically identical to *B. anthracis* and the *B. cereus* type strain ATCC 14579^T, whereas the third group combines strains of *B. thuringiensis* subsp. *morrisoni* with the *cry2* gene and strains of *B. thuringiensis* subsp. *tenebrionis* with the *cry3* gene. PCR fingerprinting with the use of six different primer systems ($(GTG)_5$, REP, ERIC, and DIR) confirmed the presence of three statistically relevant groups, whose structure correlated with that suggested by the analysis of ribosomal operon regions.

Key words: PCR fingerprinting, AR-PCR, ribosomal operon.

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The species *Bacillus thuringiensis* comprises gram-positive spore-forming bacteria characterized by the ability to produce during sporulation a complex of crystal protein toxins, also termed δ-endotoxins or Cry-proteins. These proteins exhibit highly specific insecticidal activity towards a range of pests and human parasites. The activity against larvae of insects belonging to the orders *Lepidoptera*, *Diptera*, *Coleoptera*, *Hymenoptera*, *Homoptera*, *Phthiraptera*, and *Mallophaga*, as well as other invertebrates, has been confirmed to date [1]. Due to their high specificity to particular pest species and environmental compatibility, the insecticidal crystal proteins are a valuable alternative to chemical pesticides [2]. Furthermore, cloning of the genes encoding δ-endotoxins and their expression in transgenic plants or microorganisms helps researchers tackle the problems of crop protection against pests and resistance of the latter to various pesticides [3].

Initially, all known *Bacillus thuringiensis* subspecies were defined based on insecticidal activities of the toxins encoded by the corresponding genes: thus, the proteins encoded by *cryI* genes are toxic to the order *Lepidoptera, cry2*-encoded, for *Lepidoptera-Diptera, cry3*-encoded, for *Coleoptera*, and *cry5*-encoded, for *Diptera* [4]. Presently, there are several classifications of *B. thuringiensis* strains. One of them, the so-called H-serotyping, relies on an immunological assay based on the flagellar antigen. This system classifies the known strains into more than 80 serovars [5]; however, the strains within the serovars may differ in a number of features [6]. Another classification is based on amino acid homology of insecticidal toxins encoded by *cry* or *cyt* genes. In this system, all known *cry* genes fall into 22 groups and various subgroups [7]. However, neither of the two systems reflects phylogenetic relations between the strains [8].

A variety of methods have been used to study phylogenetic relationships between the subspecies of *B. thuringiensis*. Analysis of the 16S–23S rRNA intergenic spacer sequence did not show any statistically relevant differences between the studied strains [9]. Methods based on molecular typing were also used for the investigation of phylogenetic divergence between the strains of the species in question. Application of hybridization methods, with colonies and by Southern, along with RAPD-analysis, allowed the researchers to classify the studied strains into 12 groups [10]. A study of phylogenetic relationships that relied upon restriction analysis of 16S rRNA genes identified four phylogenetic groups among subspecies of *B. thuringiensis* [6], whereas restriction analysis of 23S and 5S rRNA genes revealed ten distinct clusters [8].

The goal of the present work was to determine phylogenetic relationships between *B. thuringiensis* strains using a set of molecular biological methods, such as arbitrarily primed PCR (AP-PCR) and analysis of

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16S rRNA, the 16S–23S rRNA intergenic region, and the 5'-terminal region of 23S rRNA, often used for comparison of closely related species. AP-PCR employed primers complimentary to short repetitive sequences $(GTG)_5$ [11] and to repetitive extragenic palindromic (REP) sequences [12], as well as primers based on enterobacterial repetitive intergenic consensus (ERIC) [12] and diverged inverted repeats (DIR) [13].

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Strains of entomopathogenic bacilli of the species *B. thuringiensis* were obtained from the All-Russian Collection of Industrial Microorganisms (VKPM) and the collection of the Bioengineering Center, Russian Academy of Sciences. For the list of the studied strains, their brief characterization, and cultivation conditions, refer to [14].

DNA isolation. DNA preparations were isolated from the microbial strains by an earlier described technique [15].

Amplification, isolation, and sequencing of 16S rDNA, the 16S–23S rDNA intergenic region and 5'-terminus of 23S rDNA. The amplification was carried out on a Genius thermal cycler (Techne, Cambridge Ltd, United Kingdom) using the Bio*Taq* thermostable polymerase (Dialat Ltd, Moscow) according to the manufacturer's instructions. The universal primers 27f (5'-GTTTGATCMTGGCTCAG) and 1492R (5'-TACGGYTACCTTGTTACGACTT) were used for the amplification of 16S rDNA, and primers 1406f (5'-TGYACACACCTCCCGT) and 242r (5'-KTTCGCTCGCCRCTAC) were employed for the amplification of the 16S–23S rDNA intergenic spacer region and the 23S rDNA 5'-terminal region (Y = (C:T), M = (C:A), S = (G:C), and K = (G:T)) [16]. The time–temperature profile of PCR was as follows: the first cycle—of 3 min at 94°C, 2 min at 50°C, and 2 min at 72°C and subsequent 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gel and documented using the BioDoc Analyze system (Biometra, Germany). The fragments were extracted and purified from low-gelling-temperature agarose using the Wizard PCR Preps kit (Promega) according to the manufacturer's instructions.

Sequencing of the purified PCR fragments was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States). The nucleotide sequences were determined with a DNA Analyzer 3730 automatic sequencer (Applied Biosystems, United States). The primers used for sequencing were the same as for the PCR.

PCR fingerprinting. AP-PCR was performed using primers $(GTG)_5$ (5'-GTGGTGGTGGTGGTG), REP1-I (5'-ATGTAAGCTCCTGGGGATTCAC), REP2-I (5'-ICGICTTATCIGGCCTAC), ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC), and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG) [11, 12]. To check reproducibility of the results, all PCRs were carried out using two devices, Genius DNA thermocycler (Techne Inc., United Kingdom) and Mastercycler gradient 5331 (Eppendorf, Germany). The amplification mixture (25 μl) contained the following ingredients: 1× Bio*Taq* polymerase buffer (17 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, and 2 mM $MgCl_2$), 5 nM dNTP, 50 ng of template DNA, 12.5 pmole of each primer, and 1.25 U of Bio*Taq* DNA polymerase (Dialat Ltd, Russia). The time–temperature profile of the reaction included denaturation for 2 min at 94°C, 40 cycles of 20 s at 94°C, 30 s at 40°C, and 1 min 30 s at 72°C, and a final extension at 72°C for 7 min.

DIR-PCR used the following primer pairs: KRP2 (5'-CAGGAAGAAG) and KRP10 (5'-CTTCAAGGTT), and KRPN2 (5'-CGCCIGGIGGAT) and KRPN10 (5'-CAICICCGCCGC) [14]. The amplification was performed in a reaction mixture (25 μl) of the following composition: 1× buffer for Bio*Taq* polymerase (17 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, pH 8.8, and 6 mM $MgCl_2$), 5 nM dNTP, 50–100 ng of template DNA, 12.5 pmole of each primer, and 1.25 U of Bio*Taq* DNA polymerase (Dialat Ltd, Russia). The time–temperature profile of the reaction was as follows: the first cycle, 3 min at 94°C; 35 cycles of 30 s at 94°C, 40 s at 37°C, and 1 min at 72°C; and the final extension for 7 min at 72°C. The products of the PCR were analyzed as described above.

Phylogenetic analysis. The *de novo* determined 16S rRNA, 23S rRNA, and 16S–23S rRNA intergenic sequences of the studied *B. thuringiensis* strains were aligned with the analogous sequences of *B. anthracis, B. cereus*, and *B. thuringiensis* available from the GenBank database using the BioEdit software package [http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html]. Phylogenetic trees were constructed by the maximum parsimony method [17]. The consensus tree was generated by fusing 150 most parsimonious trees. Branches absent in more than 50 percent of trees are not shown. The phylogenetic analysis was performed using the MEGA4 software [18].

Analysis of ERIC-, REP-, $(GTG)_5$-, and DIR-PCR patterns resulted, in total, in the identification of 97 reproducible polymorphic markers. The integrated data were presented as a Jaccard pairwise genetic distance matrix, as in [21]. Phenograms were constructed using the neighbor-joining algorithm implemented in the TREECONW software package [http://bioc-www.uiaac.be/u/yvdp/treeconw.html]. Statistical significance of the trees was calculated using bootstrap analysis, by generating 500 alternative trees.

Cloning and sequencing of PCR fragments. For sequence analysis of certain specific DIR-PCR fragments, they were ligated into a pGEM-3Zf(+) vector and cloned using competent *E. coli* DH5α cells. Isola-

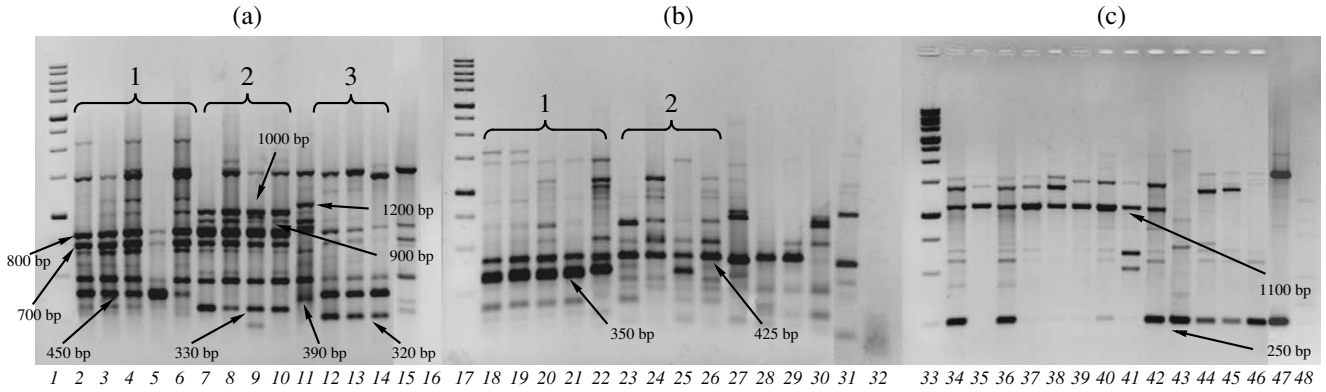

Fig. 1. Electrophoretic analysis of the PCR products obtained for DNA samples of *B. thuringiensis* strains with primers $(GTG)_5$ (a), ERIC (b), and REP (c). Lanes: *1, 17, 33*, 1 kb DNA molecular mass marker (Fermentas); *2, 18, 34*, *B. thuringiensis* subsp. *thuringiensis; 3, 9, 35*, *B. thuringiensis* subsp. *toumanoffi; 4, 20, 36, B. thuringiensis* subsp. *berliner; 5, 21, 37, B. thuringiensis* subsp. *sotto; 6, 22, 38, B. thuringiensis* subsp. *cameroun; 7, 23, 39, B. thuringiensis* subsp. *galleria; 8, 24, 40, B. thuringiensis* subsp. *dendrolimus; 9, 25, 41, B. thuringiensis* subsp. *colmeri; 10, 26, 42, B. thuringiensis* subsp. *kurstaki; 11, 27, 43, B. thuringiensis* subsp. *ostriniae; 12, 28, 44, B. thuringiensis* subsp. *morrisoni; 13, 29, 45, B. thuringiensis* subsp. *tenebrionis; 14, 30, 46, B. thuringiensis* subsp. *israelensis*; 15, *31, 47, B. mycoides* ATCC 10206; *16, 32, 48*, control PCR without template DNA. Arrows indicate characteristic PCR fragments and their sizes.

tion and purification of plasmid DNA was carried out using Wizard MiniPrep kit (Promega, United States), according to the manufacturer's instructions.

Sequencing of the cloned fragments was performed as described above, using universal plasmid-directed primers SP6 and T7.

Deposition of the nucleotide sequences. The de novo determined nucleotide sequences of 16S rRNAs, 5'-terminal regions of 23S rRNAs, and 16S–23S rRNA intergenic spacers of the studied *B. thuringiensis* strains were deposited in GenBank under the accession numbers EF117838–EF117863 and EU429660–EU429672.

RESULTS

AP-PCR-based analysis of *B. thuringiensis* strains. Various AP-PCR methods have been successfully applied for assessing the interspecies genetic relations. In this study, we used $(GTG)_5$, ERIC-, REP-, and DIR-PCR; these methods, in our case, produced the largest variety of patterns. Figure 1a shows the AP-PCR patterns obtained with $(GTG)_5$ primers. All the analyzed strains may be classified into three groups according to the patterns of their PCR fingerprints and the presence of specific fragments. The first and second groups are comprised of subspecies having the *cry1* genes but differing in the patterns of PCR fingerprints: strains of the first group exhibit a characteristic 450-bp fragment, whereas the second group is characterized by the presence of a 330-bp fragment. Furthermore, all nine strains possessing the *cry1* genes exhibit the characteristic fragments of about 700 and 800 bp. The third group includes the subspecies possessing *cry2*, *cry3*, and *cry4* genes and exhibiting a characteristic fragment of about 320 bp. The strain *B. thuringiensis* subsp. *ostriniae* CB05 differs from all the above-mentioned groups in the PCR fingerprint pattern, exhibiting specific fragments 390- and 1200-bp-long. Bands characteristic of different groups are present in the pattern of this strain, and it is hard to place it in a certain group.

The patterns obtained using ERIC and REP primers were characterized by lower strain specificity and a small number of bands (Figs. 1b, 1c). Based on these fingerprints, only the first group is clearly distinct, due to the presence of an intensive 350-bp PCR fragment.

In summary, the patterns obtained using the above-mentioned primers are not always strain-specific. Thus, several of the studied strains exhibited identical patterns. These were *B. thuringiensis* subsp. *thuringiensis* T01001 and *B. thuringiensis* subsp. *toumanoffi* B6021 (for ERIC-PCR fingerprints), and *B. thuringiensis* subsp. *dendrolimus* T04A001 and *B. thuringiensis* subsp. *kurstaki* B6066 (for $(GTG)_5$-PCR fingerprints); the patterns of *B. thuringiensis* subsp. *morrisoni* B6069 and *B. thuringiensis* subsp. *tenebrionis* B5081 were practically identical for all of the studied PCR fingerprints. The small number of bands and low specificity have earlier been noted for several AP-PCR methods [19].

Primer combinations that produced the most informative patterns in the preliminary analysis (KRP2–KRP10 and KRPN2–KRPN10) were chosen for DIR-PCR. As seen in Fig. 2, each of the studied strains demonstrated unique patterns of PCR fragments. Notably, there were common PCR fragments characteristic of all strains (e.g., the fragment of about 500 bp), fragments specific for particular groups, and individual bands distinguishing the strains. Analysis of the patterns confirms the existence of the above-described three groups revealed in the analysis with the $(GTG)_5$ primer. The composition of these groups is identical to that specified above.

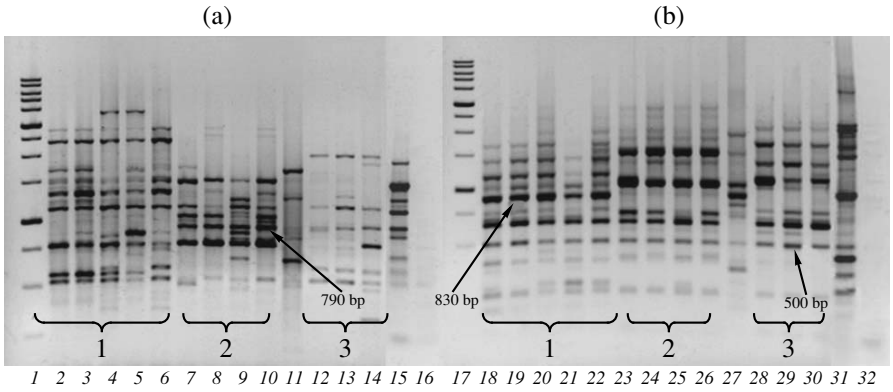

Fig. 2. Electrophoretic analysis of the PCR products obtained for DNA samples of *B. thuringiensis* strains with the primer pairs KRP2–KRP10 (a) and KRPN2–KRPN10 (b). Lanes: *1, 17*, 1 kb DNA molecular mass marker (Fermentas); *2, 18, B. thuringiensis* subsp. *thuringiensis; 3, 9, B. thuringiensis* subsp. *toumanoffi; 4, 20, B. thuringiensis* subsp. *berliner; 5, 21, B. thuringiensis* subsp. *sotto; 6, 22, B. thuringiensis* subsp. *cameroun B6775; 7, 23, B. thuringiensis* subsp. *galleria; 8, 24, B. thuringiensis* subsp. *dendrolimus; 9, 25, B. thuringiensis* subsp. *colmeri; 10, 26, B. thuringiensis* subsp. *kurstaki; 11, 27, B. thuringiensis* subsp. *ostriniae; 12, 28, B. thuringiensis* subsp. *morrisoni; 13, 29, B. thuringiensis* subsp. *tenebrionis; 14, 30, B. thuringiensis* subsp. *israelensis*; *15, 31, B. mycoides* ATCC 10206; *16, 32*, control PCR without template DNA.

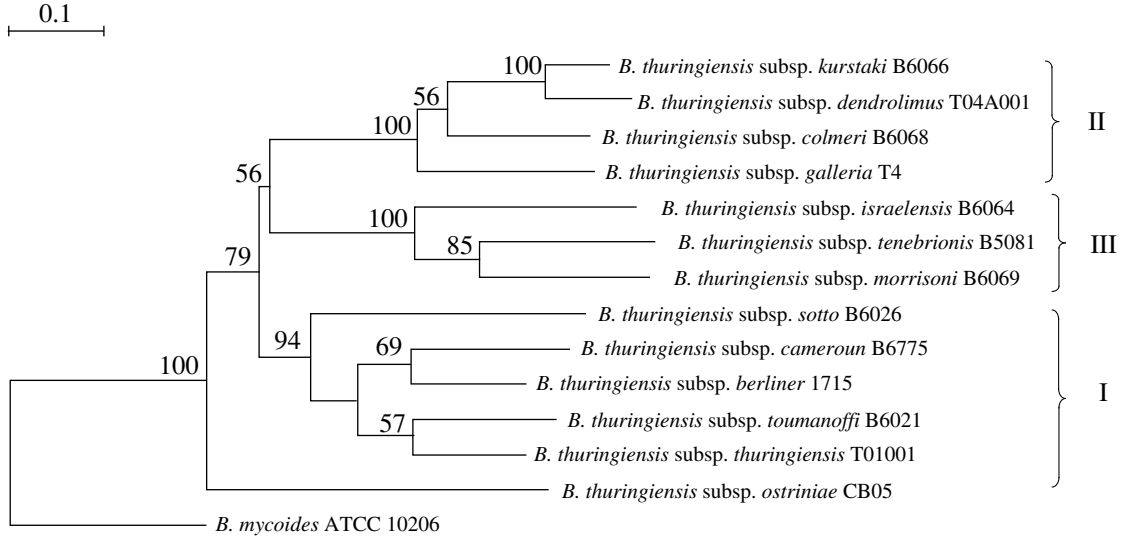

Fig. 3. Phenogram constructed based on summary analysis of AP-PCR patterns involving 97 polymorphic markers.

Figure 3 depicts a phenogram based on the summary analysis of 97 polymorphic markers. For comparison, we also used DNA of the *B. mycoides* strain ATCC 10206. The dendrogram shows that all the analyzed microorganisms, except one strain, fall into three clusters, identified with a bootstrap support of 56–99%. The first cluster (94% bootstrap) is comprised of strains *B. thuringiensis* subsp. *thuringiensis* T01001, *B. thuringiensis* subsp. *toumanoffi* B6021, *B. thuringiensis* subsp. *berliner* 1715, *B. thuringiensis* subsp. *sotto* B6026, and *B. thuringiensis* subsp. *cameroun* B6775. As mentioned above, all these strains possess *cry1* genes.

The second cluster (100% bootstrap) includes *B. thuringiensis* subsp. *galleria* CB01/T4, *B. thuringiensis* subsp. *dendrolimus* T04A001, *B. thuringiensis* subsp. *colmeri* B6068, and *B. thuringiensis* subsp. *kurstaki* B6066, also bearing *cry1* genes. The third cluster (100% bootstrap) is composed of three strains belonging to different *cry* groups: *B. thuringiensis* subsp. *morrisoni* B6069 (*cry2*), *B. thuringiensis* subsp. *tenebrionis* B5081 (*cry3*), and *B. thuringiensis* subsp. *israelensis* B6064 (*cry4*). Apart from the majority of strains (100% bootstrap) stands the *B. thuringiensis* subsp. *ostriniae* strain CB05, significantly different from other strains in the PCR patterns obtained with all primers. According to the literature data, this subspe-

Table 1. Results of homology analyses of PCR fragments

Length of the fragment, strain	GB address of the closest sequence	Degree of homology	Organism	Gene
790 bp. *B. thuringiensis* subsp. *colmeri*	gb|AE016877.1|	Identities = 769/784 (98%)	*B. cereus* ATCC14579	*microbial collagenase*
	gb|CP000903.1|	Identities = 734/787 (93%)	*B. weihenstephanensis* KBAB4	*microbial collagenase*
	gb|CP000764.1|	Identities = 496/669 (74%)	*B. cereus* subsp. *cytotoxis* NVH 391-98	*microbial collagenase*
830 bp. *B. thuringiensis* subsp. *berliner* *B. thuringiensis* subsp. *thuringiensis*	gb|AE016877.1|	Identities = 814/834 (97%)	*B. cereus* ATCC14579	*glycine oxidase*
	gb|AE017334.2|	Identities = 750/835 (89%)	*B. anthracis* str. '*Ames Ancestor*'	*glycine oxidase*
	gb|AE017355.1|	Identities = 748/836 (89%)	*B. thuringiensis* serovar *konkukian* 97-27	*glycine oxidase*

cies bears the *cry2* gene; however, its presence in the given strain was not confirmed by PCR analysis.

To reveal the nature of differences between clusters 1 and 2, bearing the *cry1* gene, some characteristic PCR fragments were cloned and sequenced. It was the 830-bp fragment for group 1 (Fig. 2b) and the 790-bp fragment for group 2 (Fig. 2a). Comparison of these sequences with the GenBank database showed that the group 1 fragments were 89–97% homologous to the gene encoding glycine oxidase, and the group 2 fragments were 74–98% homologous to the microbial collagenase gene. Both these genes are encoded by the chromosomal DNAs of *B. cereus*, *B. anthracis*, and *B. thuringiensis* strains (Table 1).

Analysis of the 16S rRNA and 5'-terminal 23S rRNA sequences and the 16S–23S rRNA spacer sequence. Analysis of various regions of the ribosomal operon is often used in the investigations of phylogenetic relationships between closely related strains. In this study, we also determined the nucleotide sequences of the 16S rRNA, as well as the 16S–23S rRNA intergenic region and the 5'-terminal region of 23S rRNA, known to be highly variable (the total length was 1875 bp). Analogous sequences of a number of *B. anthracis, B. cereus*, and *B. thuringiensis* strains, retrieved from the GenBank database, were also used for comparison. The results of the analysis revealed heterogeneity of the specified regions among the studied and reference sequences of the bacteria. Comparative analysis showed that the 16S rRNA sequences of *B. thuringiensis* strains belonging, based on AP-PCR, to the second and third groups are identical (except the sequence of subsp. *israelensis*) to the sequences of *B. anthracis* and the *B. cereus* type strain ATCC 14579^T. The strains belonging, based on AP-PCR, to the first group and *B. thuringiensis* subsp. *israelensis* had 16S rRNA sequence almost identical to that of the *B. thuringiensis* type strain ATCC 10792^T. The sequences of these strains differed from those of the first group by three nucleotide substitutions, at positions 77, 90, and 92 (numbering is by the sequence of *B. cereus* ATCC 14579^T). The fact that these substitutions have been detected in a large number of strains, including both *B. cereus* and *B. thuringiensis*, implies that their appearance was not accidental: the same alleles were found in 469 16S rRNA gene sequences of the representatives of *B. cereus* group in GenBank (Table 2).

Substitutions have been also demonstrated in the 5'-terminal region of 23S rRNA at positions 132, 157, and 174 in five strains: *B. thuringiensis* subsp. *colmeri, B. thuringiensis* subsp. *galleria*, *B. thuringiensis* subsp. *kurstaki, B. thuringiensis* subsp. *dendrolimus*, and *B. thuringiensis* subsp. *ostriniae*. As in the case of 16S rRNA, these substitutions distinguish the nucleotide sequences of the above-mentioned strains from those of the *B. thuringiensis* type strain, and are fully identical to the substitutions in the *B. anthracis* and *B. cereus* sequences. It is worth mentioning that the strain groups resulting from the analysis of 16S and 23S rRNA practically coincided, with the exception of *B. thuringiensis* subsp. *morrisoni* and *B. thuringiensis* subsp. *tenebrionis*.

Analysis of the 16S–23S rRNA intergenic region (144 bp) produced antagonistic results: the strains that, based on 16S and 23S rRNA analysis, proved identical to *B. anthracis* differed in the ITS regions. A number of *B. thuringiensis* and *B. cereus*^{T} strains exhibited a single-nucleotide deletion at position 32. Substitutions were also detected at positions 133 and 135 in strains of *B. thuringiensis* subspp. *sotto* and *israelensis*, as well as in a number of reference strains. However, these substitutions apparently occur at random.

Table 2 illustrates the distribution analysis of polymorphic alleles in the sequences of ribosomal loci of the "*B. cereus*" group available in GenBank. Each of the detected alleles was found in a number of species, with rather significant differences in the occurrence rates. For example, the alleles located in the 5'-terminal region of 16S rRNA (A/T/T) at positions 77, 90, and 92 (numbering by the sequence of *B. cereus* ATCC 14579^T) and in the 5'-terminal region of 23S rRNA (C/T/A) at positions 132, 157, and 174 were more typ-

Table 2. Occurrence rate of certain alleles pertaining to regions of the ribosomal operon (16S and 5'-23S rRNA and ITS) in 677 sequences belonging to representatives of the *Bacillus cereus* group available in GenBank

Locus	Allele	Signature	Occurrence frequency of the allele, %					Total		Representative species of the genus *Bacillus* for each allele	Total for the representative species, %
			Bc*	Bt	Ba	Bm	Bsp	%	Number		
5'-16S	I	ATT	27.72	7.68	24.09	2.77	37.74	100	469	*anthracis/cereus*	51.8
	II	GCA	14.88	13.22	1.65	0.00	70.25	100	121	*cereus/thuringiensis*	28.1
5'-23S	I	CTA	62.16	13.51	24.32	0.00	0.00	100	37	*anthracis/cereus*	86.48
	II	TCT	36.36	27.27	0.00	36.36	0.00	100	11	*cereus/thuringiensis/mycoides*	100.00
ITS	I	ATA	25.00	16.67	41.67	4.17	12.50	100	24	*anthracis/cereus/thuringiensis*	83.3
	II	A-A	73.33	26.67	0.00	0.00	0.00	100	15	*cereus/thuringiensis*	100.0

* Bc – *B. cereus*, Bt – *B. thuringiensis*, Ba – *B. anthracis*, Bm – *B. mycoides*, Bsp – unidentified *Bacillus* species.

ical of *B. anthracis*. Both variants occurred in *B. cereus* strains. The alternative alleles for both regions were more common for sequences of the species *B. thuringiensis* and *B. mycoides*. Alleles located on 23S rRNA and on ITS were more group-specific as compared to those located on 16S rRNA; the alleles T/C/T for 23S rRNA and A/-/A for ITS were not found in the sequences of *B. anthracis*. In general, no strong correlation was revealed between these alleles and particular species.

The phylogenetic tree constructed based on united sequences of the 16S rRNA, ITS, and 23S rRNA 5'-terminal region, with the total length of 1875 bp, is presented in Fig. 4. All the studied *B. thuringiensis* strains

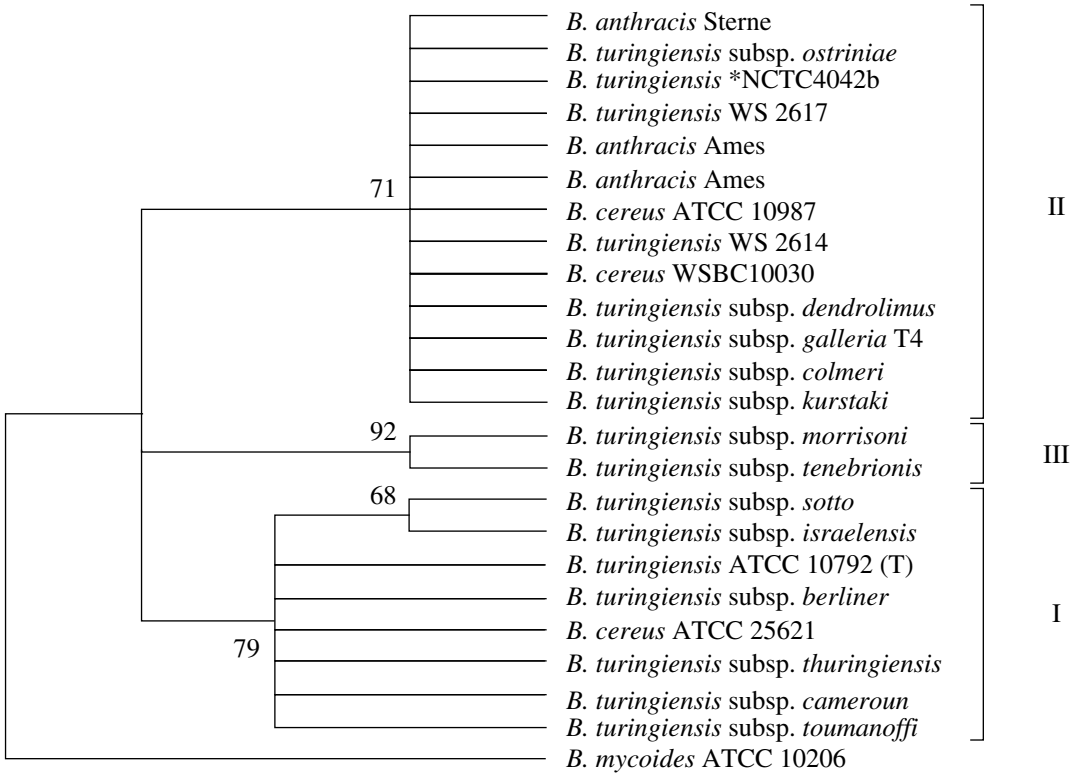

Fig. 4. Phylogenetic tree constructed based on united sequences of 16S rRNA, ITS and the 23S rRNA 5'-terminal region.

fell into much the same groups as they did based on the AP-PCR analysis (Fig. 3). Exceptions were the strains of the subspecies *B. thuringiensis* subsp. *ostriniae* and *israelensis*. The strain *B. thuringiensis* subsp. *ostriniae* CB05, which was ranked as a separate branch based on AP-PCR, fell into the second (*cry1*) group based on the sequence analysis. The strain of *B. thuringiensis* subsp. *israelensis*, which was placed in the third group based on AP-PCR, proved to be closer to the first group according to results of analysis of the variable regions of rRNA genes. Otherwise, the trees had the same topologies. The reference strains of *B. cereus* and *B. anthracis* grouped together with the representatives of *B. thuringiensis* subgroups 1 and 2, the first subgroup containing the *B. cereus* strain ATCC 25621 and the *B. thuringiensis* strain ATCC 10792^T. Other strains, including *B. cereus* ATCC 14579^T and *B. anthracis* representatives, were placed in the second subgroup. Thus, signature analysis of hypervariable regions of the ribosomal operon also subdivided the studied strains into several groups; however, inside each group, these strains were indistinguishable.

DISCUSSION

B. thuringiensis belongs to the *B. cereus* group, which also includes the species *B. anthracis, B. cereus*, and *B. mycoides* [20]. Their chromosomes exhibit a high level of gene synteny and DNA homology, with a limited difference in the gene composition [21]. Phylogenetic relations between the species and strains of this group were studied based on the nucleotide sequence analysis of 16S rRNA, *gyrB*, *rpoC* [9, 22, 23], and AFLP fingerprinting [24]. Several groups were identified that included both *B. cereus* and *B. thuringiensis* strains.

Our analysis of the ribosomal operon variable regions also showed that the group structure strongly depends on the region under study, e.g., 16S rRNA or ITS. Besides, the analysis of individual regions revealed only two types of nucleotide sequences, while a combined analysis subdivided the studied sequences into three groups. Thus, the investigation of several variable regions within the ribosomal operon allowed us to obtain more adequate results. Obviously, more groups exist, but the selection of the strains was limited in our study.

In contrast to the locus-specific differences revealed by the analysis of the ribosomal operon, PCR fingerprinting indicates unanimous differences between closely related genomes. The sensitivity of PCR fingerprinting is known to depend on the primer choice. On the whole, all AP-PCR techniques employed demonstrated statistically relevant differences between the studied strains of *B. thuringiensis.* The conducted analysis separated the strains into three or four groups, dependent on the method used.

ERIC- and REP-PCR, which use primers complimentary to specific intergenic regions of bacterial genomes and the $(GTG)_5$ primers, representing simple tandem repeats common to certain genes and intergenic sequences, provide for the analysis of short and conservative genome regions and show low correlation with other methods of genotype comparison. For this reason, the patterns obtained by such PCR methods display a relatively large number of common bands, and thus do not always allow for identification of groups (as is the case with REP primers). The sequences homologous to DIR primers are not associated with a particular conserved region of the genome; they are evenly distributed among the protein-coding DNA fragments. As a result, they produce patterns that allow detection of minor differences between microbial strains. Such a high specificity of patterns obtained with DIR primers opens the possibility to make preliminary conclusions about distinctions between bacterial strains without employing other analyses. The clustering based on fingerprints obtained with these primers, was practically identical to that provided by the analysis of variable regions of the ribosomal operons.

The fact that the strains bearing *cry1* genes fell into two groups apparently points to genome-level differences and is not associated with the plasmids where these genes are located. This assumption is confirmed by the fact that the PCR fragments characteristic for each of these groups were located in conservative regions of chromosomal DNA, in particular, the loci of glycine oxidase and microbial collagenase genes.

Our analysis revealed no correlation between the phenetic groups of *B. thuringiensis* and the associated types of *cry* genes: in some cases strains with the same type of *cry* genes were to be placed into different groups (*cry1* and *cry2*), and in other cases strains with different *cry* types formed one group (*cry2* and *cry3*). Since the methods used allowed us to compare regions of genomic DNA, not plasmids bearing *cry* genes, the results reflect phylogenetic distinctions between the studied strains. The changes in relations between the strains belonging to the third cluster (*B. thuringiensis* subsp. *tenebrionis* B5081 clustered with *B. thuringiensis* subsp. *israelensis* B6064, not with *B. thuringiensis* subsp. *morrisoni* B6069 as in the case of the dendrogram based on DIR patterns) are attributable to the limited selection of strains representing the *cry2, cry3*, and *cry4* groups.

Nevertheless, both of the analysis methods employed suggested the existence of several phylogenetic groups within the studied set of strains. These groups relate equally to *B. thuringiensis* and *B. cereus*. These findings once again raise the question of their taxonomic status.

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