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EXPERIMENTAL ARTICLES

Use of DIR-PCR for Elaboration of Molecular Markers of Intraspecies Bacterial Groups as Exemplified by *Bacillus thuringiensis*

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Abstract—A recently developed PCR-fingerprinting method, the so-called DIR (diverged inverted repeats)-PCR, was used for quick search for molecular markers of *Bacillus thuringiensis* subspecies carrying the *cry1* genes. The analysis of the fingerprints obtained with this method made it possible to reveal PCR fragments characteristic of the subspecies that produce proteins toxic for insects of the order Lepidoptera. Cloning and sequencing of these fragments allowed systems of SCAR (sequence characterized amplified region) primers to be designed, which are specific to the above group of *B. thuringiensis* strains. Comparison of the specific fragments with sequences available in the GenBank database revealed their homology with the *rpoC* gene family and the adjacent spacer region, suggesting chromosomal localization of these markers. This increases the reliability of the designed system of SCAR primers, because plasmids may be lost or transferred by transformation between closely related strains. It was demonstrated that the DIR-PCR method allows markers to be elaborated that are linked to diagnostic genotypic and phenotypic characteristics of bacteria.

Key words: bacilli, genomic fingerprints, PCR, SCAR primers.

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Bacillus thuringiensis is a species of gram-positive spore-forming bacteria. Its characteristic feature is the capacity to produce a complex of crystal protein toxins referred to as δ-endotoxins or Cry proteins. These proteins exhibit insecticidal activity specific to a number of agricultural pests and human parasites. To date, activity against invertebrates of the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera (Mallophaga), Acari, Nemathelminthes, Platyhelminthes, and Sarcomastigorphora has been shown [1]. Due to the high specificity to pests, rare occurrence of acquired resistance in target organisms, and safety for other groups of animals, insecticidal crystal proteins are a valuable alternative to chemical pesticides [2].

Moreover, cloning and expression of δ-endotoxin-encoding genes (*cry* genes) in transgenic plants or microorganisms help to solve the problem of agricultural plant protection from pests [3].

Many years' research has led to the isolation and description of more than 50 thousand strains of these bacteria [4]. Based on H-serotyping, *B. thuringiensis* strains were subdivided into more than 80 serovars [5], whereas the nomenclature based on the similarity of *cry* genes divided them into 22 types and further subdivided into subtypes [6]. The phylogenetic relationships between *B. thuringiensis* strains were studied on the basis of comparison of various ribosomal operon regions, as well as by RAPD-analysis, and revealed among them several groups that do not coincide with the *cry* types [4, 7, 8].

When new strains are isolated, it is important to determine their insecticidal activity. For this purpose, hybridization with probes for the already-known *cry* genes in search for their homologues [9], analysis with the use of monoclonal antibodies [10], or determination of the serovar of crystal proteins [11] are carried out. Unfortunately, these methods are insufficient to accurately predict insecticidal activity or are too costly and laborious for routine analyses.

Preliminary assessment of new strains has also been made by PCR with primers homologous to conservative sites of the *cry* genes, which allows the families of these genes to be quickly identified in the strains analyzed [12]. Such a method of analysis is limited to detecting only already known genes; therefore, new approaches are being developed to reveal new types of *cry* genes, e.g., E-PCR, which implies two-stage PCR: with degenerated primers and then with primers specific to a particular *cry* gene [13]. However, the elaboration of such detection systems requires knowledge of the primary structure of the genes analyzed.

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Species and strains of bacilli used in this work

Microorganisms studied	Types of *cry* genes revealed	Target insects
B. thuringiensis subsp. *berliner* 1715	*cry1*	Lepidoptera
B. thuringiensis subsp. *colmeri* B6068	*cry1*, *cry2*	Lepidoptera
B. thuringiensis subsp. *cameroun* B6775	*cry*1	Lepidoptera
B. thuringiensis subsp. *galleria* CB01/T4	*cry1*, *cry2*	Lepidoptera
B. thuringiensis subsp. *galleria* B-696	*cry1*	Lepidoptera
B. thuringiensis subsp. *dendrolimus* CB02	*cry1*	Lepidoptera
B. thuringiensis subsp. *kurstaki* B6066	*cry1*	Lepidoptera
B. thuringiensis subsp. *sotto* B6026	*cry1*	Lepidoptera
B. thuringiensis subsp. *thuringiensis* CB03	*cry1*	Lepidoptera
B. thuringiensis subsp. *toumanoffi* B6021	*cry1*	Lepidoptera
B. thuringiensis subsp. *morrisoni* B6069	*cry1*, *cry2*	Lepidoptera/Diptera
B. thuringiensis subsp. *ostriniae* CB05	*cry2**	Lepidoptera/Diptera
B. thuringiensis subsp. *tenebrionis* B5081	*cry3*	Coleoptera
B. thuringiensis subsp. *israelensis* B6064	*cry4*	Diptera

* The type of the *cry* gene is indicated according to [20].

The genomic fingerprinting methods, which allow minor differences between individual strains or groups of strains to be revealed, are an alternative approach to the search for molecular markers of different groups of microorganisms. In particular, DIR-PCR [14] has been successfully used for analyzing geographical isolates of *Xanthomonas campestris* and elaborating SCAR markers for this species, as well as for revealing genomic differences between dissociants in *B. cereus* and *B. subtilis* [15, 16].

The aim of this work was to use DIR-PCR to search for markers that would allow natural populations to be screened for *cry1*-containing strains of *B. thuringiensis*. This approach allows potential markers to be revealed without knowing the primary structure of the genomes of the target microorganisms.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. This work used strains of *Bacillus thuringiensis* from the Russian National Collection of Industrial Microorganisms (VKPM) and the collection of the Bioengineering Center, Russian Academy of Sciences, as well as the reference strains *B. thuringiensis* 4Q281, *B. cereus* NCTC 9620, *B. cereus* B-16, *B. mycoides* ATCC 10206, and *B. subtilis* 720 from the collection of the Bioengineering Center, Russian Academy of Sciences.

The list and some characteristics of *B. thuringiensis* strains are presented in the table. All the microorganisms were grown on agarized LB medium at 37°C [17].

DNA isolation. DNA from the bacterial strains was isolated as described in [18].

PCR analysis of *cry* genes in *B. thuringiensis* strains. The analysis of the strains for the presence of particular *cry* genes was carried out by PCR with earlier described primers: for *cry1* genes, $I^{(-)}$ 5'-MDATY-TCTAKRTCTTGACTA-3' and $I^{(+)}$ 5'-TRACRHTD-DBDGTATTAGAT-3' [13]; Lep1F 5'-CCGGTGCTG-GATTTGTGTTA-3' and Lep1R 5'-AATCCCGTAT-TGTACCAGCG-3'; Lep2F 5'-CCGAGAAAGT-CAAACATGCG-3' and Lep2R 5'-TACATGCCCTTT-CACGTTCC-3' [12]; for *cry2* genes, $II^{(+)}$ 5'-TAAA-GAAAGTGGGGAGTCTT-3' and $II^{(-)}$ 5'-AACTC-CATCGTTATTTGTAG-3' [19]; for *cry3* genes, Col1F 5'-GTCCGCTGTATATTCAGGTG-3' and Col1R 5'-CA-CTTAATCCTGTGACGCCT-3'; Col2F 5'-AGGTGC-CAACTAACCATGTT-3' and Col2R 5'-GATCCTAT-GCTTGGTCTAGT-3' [12]; for *cry4* genes, Dip1F 5'-CAAGCCGCAAATCTTGTGGA-3' and Dip1R 5'-ATTGCTTGTTTCGCTACATC-3'; Dip2F 5'-GGT-GCTTCCTATTCTTTGGC-3' and Dip2R 5'-TGAC-CAGGTCCCTTGATTAC-3' [12].

Amplification with all the primer pairs, except the $I^{(-)}$–$I^{(+)}$ pair, was carried out in 25 ml of amplification mixture of the following composition: 1× BioTaq polymerase buffer (Promega, United States); 1.5 mM $MgCl_2$; dNTPs, 5 nmol each; template DNA, 50 ng; specific primers, 25 pmol; and BioTaq DNA polymerase (Dialat LTD, Russia), 1.25 units. For the $I^{(-)}$–$I^{(+)}$ primer pair, the $MgCl_2$ concentration was 3 mM. The time–temperature profile for most primer pairs was as follows: 94°Cfor 5 min; then, 30 cycles of 94°C for 1 min, 43°C for 45 s, and 72°C for 2 min; and final extension, 72°C for 7 min. The Lep2F–Lep2R and Dip1F–Dip1R primer pairs, for which the annealing temperature was 53 and 58°C, respectively, were an exception. The PCR products were analyzed by electro-

phoresis in 1% agarose gel, and the results were documented using the BioDocII system of gel documentation (Biometra, Germany).

PCR-fingerprinting. DIR-PCR was carried out on a Cetus 480 (Perkin Elmers, Sweden) device using the primers KRPN1 5'-TCIIAAGCTTCA-3', KRP2 5'-CAGGAAGAAG-3', KRPN2 5'-CGCCIGGIGGAT-3', KRP8 5'-GAAGTTCAGG-3', and KRP10 5'-CTTCAAGGTT-3', where I is inosine. The amplification mixture (25 μl) was of the following composition: 1× BioTaq polymerase buffer (17 mM $(NH_4)_2SO_4$; 67 mM Tris–HCl, pH 8.8; 6 mM $MgCl_2$); dNTPs, 5 nmol each; template DNA, 50 ng; primers, 25 pmol each; and BioTaq DNA polymerase (Dialat LTD, Russia), 1.25 units. The time–temperature profile was as follows: primary denaturation, 94°C for 3 min; then, 35 cycles of 94°C for 30 s, 37°C for 40 s, and 72°C for 1 min; and final extension, 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1% agarose gel, and the results were documented using the BioDocII system of gel documentation (Biometra, Germany).

Cloning, sequencing, and analysis of PCR fragments. PCR fragments were isolated from low-gelling-temperature agarose and purified using a Wizard PCR Preps kit (Promega) according to the manufacturer's recommendations. The purified fragments were cloned in the pGEM-3Zf(+)vector in competent cells of *E. coli* DH5α. The isolation and purification of the plasmid DNA were carried out using a Wizard MiniPrep kit (Promega) according to the manufacturer's recommendations.

The cloned PCR fragments were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, United States). The nucleotide sequences were determined on an automatic ABI PRISM 3100 sequencer (Applied Biosystems) using the universal plasmid primers SP6 and T7.

The comparison of the nucleotide sequences of the marker fragments with the sequences available in the GenBank database was carried out using the NCBI BLAST program [http//www.ncbi.nlm.nih.gov/blast].

SCAR-PCR. The marker fragments were amplified from DNA of entomopathogenic *B. thuringiensis* strains and reference strains of other *Bacillus* species using the de novo designed SCAR-primers F2 5'-CAGGCAGTTATCAGTGAAATC-3', F3 5'-AAAGTAACTCCAGGACAGCC-3', and R3 5'-CTGTTCCACATAAATCTCATC-3' on a Cetus 480 (Perkin Elmers, Sweden) device or a Genius thermal cycler (Techne (Cambridge) LTD, United Kingdom). The reaction mixture (25 μl) was of the following composition: 1× BioTaq polymerase buffer with $(NH_4)_2SO_4$ (Fermentas, Lithuania); 1 mM $MgCl_2$; dNTPs, 7.5 nmol each; template DNA, 50 ng; primers, 12.5 pmol each; and BioTaq DNA polymerase (Dialat LTD, Russia), 1.25 units. The time–temperature profile was as follows: primary denaturation, 94°C for 3 min; then, 27 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The PCR products were analyzed by electrophoresis and the results were documented as described above.

RESULTS AND DISCUSSION

As a result of screening the collection of *B. thuringiensis* strains maintained at the Bioengineering Center, Russian Academy of Sciences, we selected those in which the presence of the corresponding *cry* genes was confirmed by PCR analysis with the use of primers reported to be specific to different types of the *cry* genes. The results of this analysis are shown in Fig. 1. In strain *B. thuringiensis* subsp. *ostriniae* CB05, the presence of *cry2* genes was established earlier from the type of the δ-endotoxin crystals produced [20]. Our PCR analysis revealed none of the four types of *cry* genes in this strain; nevertheless, this strain was included in further analyses as a reference strain. In strains *B. thuringiensis* subsp. *morrisoni* B6069, *B. thuringiensis* subsp. *colmeri* B6068, and *B. thuringiensis* subsp. *galleria* CB01/T4, PCR analysis revealed the simultaneous presence of the *cry1* and *cry2* genes, which agrees with data of other researchers [12, 21].

The strains producing δ-endotoxins of different types, selected as a result of screening, were used for primary assessment of polymorphism among *B. thuringiensis* subspecies. For this analysis, DIR-PCRs were performed with single primers and their paired combinations (data not shown). Eventually, the DNA-fingerprints obtained with the use of the KRP8–KRP10 primer pair were chosen for further work. The patterns obtained with this primer pair did not exhibit pronounced strain specificity; however, a PCR fragment approximately 810 bp in size was peculiar to all strains producing the toxins pathogenic to lepidopteran insects and possessing *cry1* genes (Fig. 2). This fragment was used for elaborating molecular markers specific to strains possessing *cry1* genes.

In order to unravel the nature of the specific marker KRP-810 revealed with the KRP8–KRP10 pair of primers, PCR fragments of two *B. thuringiensis* strains (*B. thuringiensis* subsp. *berliner* 1715 and *B. thuringiensis* subsp. *thuringiensis* CB03) were cloned in the vector pGEM-T. Five clones of each of the strains were selected for sequence analysis. The length of the nucleotide sequences obtained was 813 bp, all the sequences being virtually identical.

The comparison of the nucleotide sequence of the KRP-810 fragment with sequences available in the GenBank database revealed a high degree of homology between the studied fragment and the sequences of the *rpoC* genes (encoding the RNA polymerase β-subunit) and adjacent intergenic regions: with strains *B. cereus* ATCC 10987 (AE017194) and ATCC 14579 (AE016877), the homology was 96.6%; with strains *B. anthracis* Ames (AE016879) and Sterne (AE017225), it was 95.9%. With another *B. thuringiensis* strain represented in the GenBank, *B. thuringiensis*

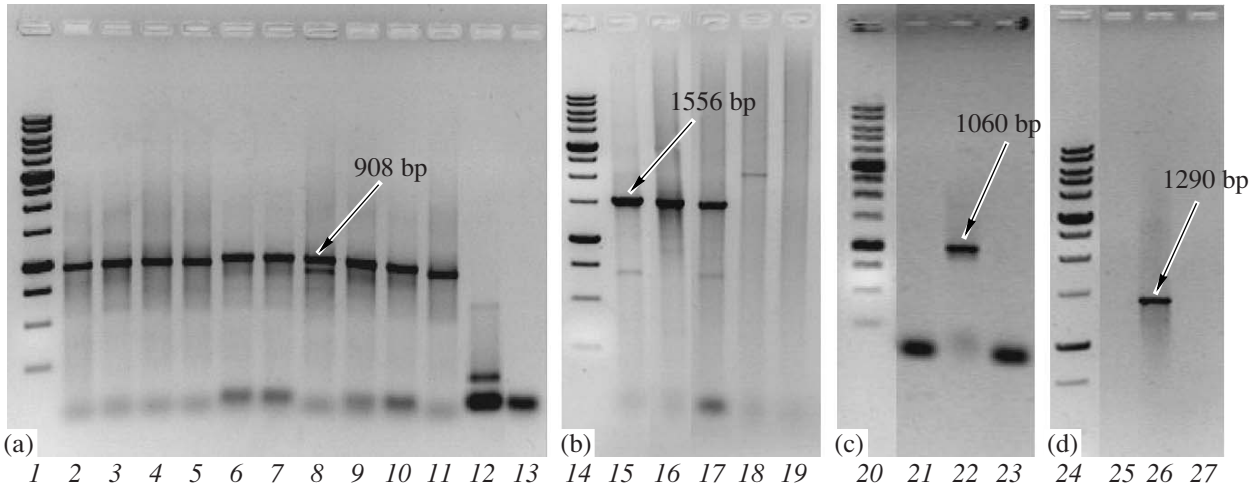

Fig. 1. PCR analysis of *B. thuringiensis* strains for the presence of particular *cry* genes with primers (a) Lep2F-Lep2R, (b) $II^{(+)}$–$II^{(-)}$, (c) Col2F-Col2R, and (d) Dip2F-Dip2R. Lanes: (*1*, *14*, *20*, *24*) the DNA 1 kb molecular mass marker (Fermentas, Lithuania); *2*, *15* – *B. thuringiensis* subsp. *colmeri*; (*3*) *B. thuringiensis* subsp. *dendrolimus* CB02; (*4, 16*) *B. thuringiensis* subsp. *galleria*; (*5*) *B. thuringiensis* subsp. *kurstaki*; (*6*) *B. thuringiensis* subsp. *berliner*; (*7*) *B. thuringiensis* subsp. *cameroun*; (*8*) *B. thuringiensis* subsp. *sotto*; (*9*) *B. thuringiensis* subsp. *thuringiensis*; (*10*) *B. thuringiensis* subsp. *toumanoffi*; (*11, 17*) *B. thuringiensis* subsp. *morrisoni*; (*12, 18, 21, 25*) *B. thuringiensis* subsp. *ostriniae*; (*22*) *B. thuringiensis* subsp. *tenebrionis*; (*26*) *B. thuringiensis* subsp. *israelensis* B6064; (*13, 19, 23, 27*) PCR in the absence of template DNA. The arrows indicate fragments of *cry* genes.

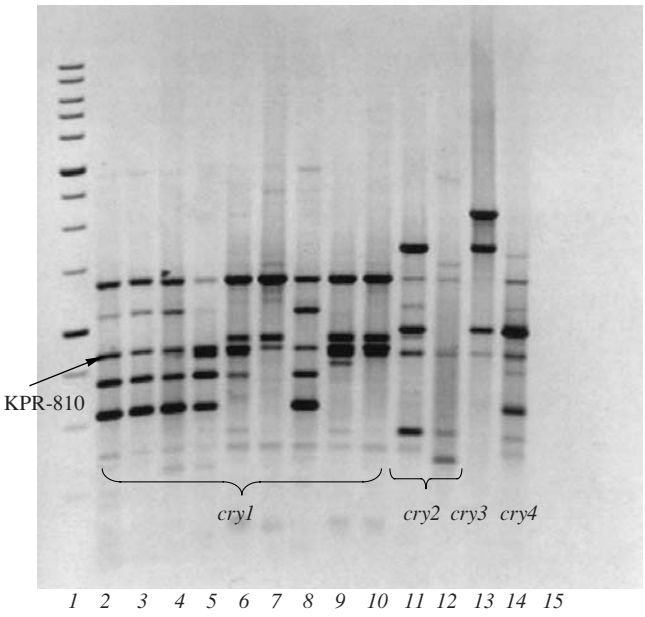

Fig. 2. DIR-PCR using the KRP8-KRP10 primers. Lanes: (*1*) the DNA 1 kb molecular mass marker (Fermentas, Lithuania); (*2*) *B. thuringiensis* subsp. *thuringiensis*; (*3*) *B. thuringiensis* subsp. *toumanoffi*; (*4*) *B. thuringiensis* subsp. *berliner*; (*5*) *B. thuringiensis* subsp. *sotto*; (*6*) *B. thuringiensis* subsp. *galleria*; (*7*) *B. thuringiensis* subsp. *dendrolimus*; (*8*) *B. thuringiensis* subsp. *cameroun*; (*9*) *B. thuringiensis* subsp. *colmeri*; (*10*) *B. thuringiensis* subsp. *kurstaki*; (*11*) *B. thuringiensis* subsp. *morrisoni*; (*12*) *B. thuringiensis* subsp. *ostriniae*; (*13*) *B. thuringiensis* subsp. *tenebrionis*; (*14*) *B. thuringiensis* subsp. *israelensis*; (*15*) PCR in the absence of template DNA. The arrow indicates the Lepidoptera-specific PCR fragment.

subsp. *konkukian* 9727 (AE017355), the homology was also 95.9%. A relatively high homology (83%) was also revealed with a *rpoC* fragment of *B. subtilis* 168 (D64127).

As a result of the above comparative analysis, specific primers for the most variable regions were designed (forward primers F2 and F3 and reverse primer R3). However, due to the high degree of conservation of the nucleotide sequences of the KRP-810 fragment and of the homologous regions of *rpoC* of *B. anthracis, B. cereus*, and *B. thuringiensis* subsp. *thuringiensis*, the designed SCAR primers differ in only one to three positions. Taking this into account, sufficiently stringent conditions—a high annealing temperature and a low $MgCl_2$ concentration—were used in PCRs with these SCAR primers.

The amplification with the proposed primer pairs (F2–R3 and F3–R3) revealed specific PCR fragments (705 and 579 bp, respectively) in all *B. thuringiensis* strains producing toxins against Lepidoptera (Fig. 3). Such bands were not revealed in other species of bacilli (*B. cereus*, *B. subtilis*, *B. licheniformis*, and *B. mycoides*) or in strains of *B. thuringiensis* belonging to other *cry* groups, or in strains in which *cry* genes have not been determined.

That the fragment specific to the *cry1*-possessing strains has chromosomal and not plasmid localization needs special discussion. It has already been mentioned above that, based on the analysis of various regions of the ribosomal operon, *B. thuringiensis* strains are subdivided into several phylogenetic groups, but this subdivision does not correlate with the types of *cry* genes

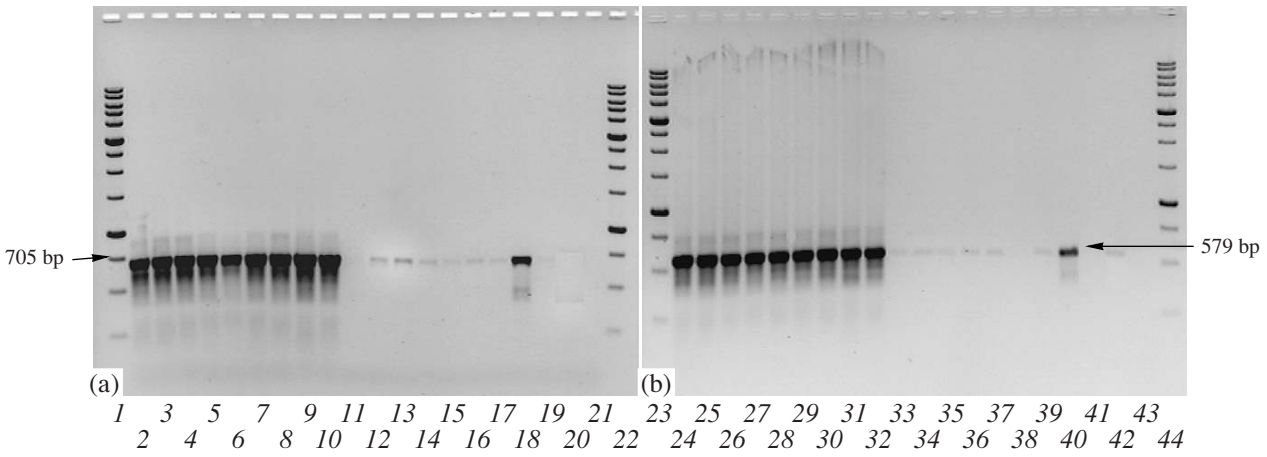

Fig. 3. Testing of the designed SCAR primer systems (a) F2–R3 and (b) F3–R3. Lane: (*1, 22, 23, 44*) the DNA 1 kb molecular mass marker (Fermentas, Lithuania); (*2, 24*) *B. thuringiensis* subsp. *thuringiensis*; (*3, 25*) *B. thuringiensis* subsp. *toumanoffi*; (*4, 26*) *B. thuringiensis* subsp. *berliner*; (*5, 27*) *B. thuringiensis* subsp. *galleria*; (*6, 28*) *B. thuringiensis* subsp. *sotto*; (*7, 29*) *B. thuringiensis* subsp. *dendrolimus*; (*8, 30*) *B. thuringiensis* subsp. *cameroun*; (*9, 31*) *B. thuringiensis* subsp. *colmeri*; (*10, 32*) *B. thuringiensis* subsp. *kurstaki*; (*11, 33*) *B. thuringiensis* subsp. *morrisoni*; (*12, 34*) *B. thuringiensis* subsp. *ostriniae*; (*13, 35*) *B. thuringiensis* subsp. *tenebrionis*; (*14, 36*) *B. thuringiensis* subsp. *israelensis*; (*15, 37*) *B. cereus* NCTC 9620; (*16, 38*) *B. cereus* B-16; (*17, 39*) *B. thuringiensis* 4Q281; (*18, 40*) *B. thuringiensis* subsp. *galleria* B-696; (*19, 41*) *B. mycoides* ATCC 10206; (*20, 42*) *B. subtilis* 720; (*21, 43*) PCR in the absence of template DNA. The arrows indicate the corresponding marker fragments.

they possess. The coincidence revealed by us between the type of *cry* genes and certain signatures in the *rpoC* sequence may be accidental and be determined by the limited number of the strains used in this work; however, it is necessary to point out that *rpoC* is one of the genes used for taxonomic analysis, and its sequence carries a significant phylogenetic message suitable for intraspecies differentiation of bacteria [22]. Thus, the approach used by us demonstrates the possibilities of using DIR-PCR for quick search for specific markers for various groups of microorganisms.

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