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PRODUCERS, BIOLOGY, SELECTION, AND GENETIC ENGINEERING

Design of a PCR Test Based on the *gyrA* Gene Sequence for the Identification of Closely Related Species of the *Bacillus subtilis* Group

L. N. Borshchevskaya, A. N. Kalinina, and S. P. Sineokii

State Research Institute for Genetics and Selection of Industrial Microorganisms (GosNIIgenetika), Moscow, 117545 Russia
e-mail: larbor3@rambler.ru

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Abstract—A method for the taxonomic identification of seven closely related bacterial species of the *Bacillus subtilis* group (*B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. vallismortis*, *B. atrophaeus*, *B. sonorensis*, and *B. mojavensis*) using specific primers selected on the basis of the *gyrA* gene sequences was developed. The effectiveness of this method both for the identification of pure cultures of type strains of this group and for the precise species identification of collection and industrial bacterial strains was demonstrated. The principal possibility of using this method for detecting *B. subtilis* group bacteria in mixed cultures was shown.

Keywords: *B. subtilis*, *gyrA* gene, identification, species-specific primers

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Strains of microorganisms belonging to the genus *Bacillus* are commonly used as probiotics in the correction of dysbiotic disorders of the gastrointestinal tract and are components of many medicines for humans and livestock. Bacillar strains are also used for soil remediation (e.g., for oil biodegradation in soil), for protection of plants against phytopathogens, as producers of many extracellular enzymes (e.g., proteases, xylanases, and amylases), etc.

The most valuable probiotic properties are exhibited by bacteria belonging to the *B. subtilis* group. This group includes *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. vallismortis, B. atrophaeus, B. sonorensis*, and *B. mojavensis*. These species share a great similarity at the phenotypic level. Currently, species identification of bacilli of the *B. subtilis* group is primarily based on morphological differences, the use of diagnostic media, and biochemical analysis. These tests are time-consuming and often give ambiguous results, especially in the determination of closely related species. For example, the fatty acid composition of the biomass is the only phenotypic trait that makes it possible to reliably distinguish *B. mojavensis* and *B. vallismortis* from each other and from *B. subtilis* [1, 2].

The lack of diagnostic, phenotypic, and biochemical characteristics make molecular methods for the identification of species of the *B. subtilis* group the most relevant. Currently, the most accurate and versatile method for the identification of different organisms is the analysis of nucleotide sequences of ribosomal RNA genes [3–5]. However, in the case of identification of bacteria of the *B. subtilis* group, this method does not allow for accurate species identification because of the high homology of the nucleotide sequences of the ribosomal genes [6] and should be complemented by other tests.

The high homology level of the ribosomal genes of organisms from the *B. subtilis* group also makes inefficient the identification of bacteria by the 16S RNA gene sequence [7].

An analysis of published data showed that the target genes for additional species-specific identification of closely related organisms are most commonly represented by operational (the so-called housekeeping) genes, which are more susceptible to mutational variations and the products of which are most important for the metabolism of microorganisms of this taxon. The differences in the nucleotide composition of these genes allow for an accurate species identification of genetically related species of microorganisms. It was suggested to use the *secY* and *rpoB* genes as targets for distinguishing between the species *B. subtilis, B. icheniformis*, and *B sonorensis* [8] and the *gyrB* gene for distinguishing between the species *B. amyloliquefaciens* and *B. subtilis* [9]. The authors of [10] describe the use of the *gyrA* gene sequence for the phylogenetic analysis of bacteria of the *B. subtilis* group.

Abbreviations: LB medium, Luria–Bertani medium; PCR, polymerase chain reaction; T, type strain; EDTA, ethylenediamine acid; dNTP, deoxynucleoside triphosphates.

Table 1. Bacterial strains used in the study

Species name	Code designation of a strain in collections	Note
B. subtilis	DSM 10, ATCC 6051, VKPM B-9865	Type strain
B. licheniformis	14580, DSM 13, NCIB 9375, NCTC 10341, NRRL NRS-1264, VKPM B-10956	To ace
B. amyloliquefaciens	DSM 7, ATCC 23350,VKPM B-9866	"
B. sonorensis	DSM 13779, NRRL B-23154, VKPM B-l 1122	"
B. atrophaeus	DSM 7264, ATCC 49337, NRRL- NRS 213, VKPM B-l 1116	"
B. mojavensis	DSM 9205, ATCC 51516, NRRL B-14698, VKPM B-l 1119	"
B. vallismortis	DSM 11031, NRRL B-14890, VKPM B-l 1123	"
B. sonorensis	VKPM B-1517	Collection strain
B. amyloliquefaciens	VKPM B-2984	To ace
B. subtilis	VKPM B-1283	"

In all these studies, to accurately distinguish between closely related species on the basis of operational gene sequences, it is intended to use gene cloning, restriction analysis or sequencing of PCR fragments, or DNA–DNA hybridization, which makes such an analysis much more expensive and laborious. In addition, none of these studies proposed a universal method for a rapid and reliable determination of the specific affiliation of all species of bacteria in the *B. subtilis* group.

The goal of this study was to develop a method for the identification of seven species of genetically closely related species of microorganisms from the *B. subtilis* group on the basis of differences in the nucleotide composition of the *gyrA* gene, encoding the alpha subunit of DNA gyrase.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are summarized in Table 1.

Conditions for the cultivation of bacterial strains. Bacteria were grown in an LB medium containing 10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride (reagent grade) (Khimmed, Russian), and 20 g of agar (Rapgeas, Spain) in 1 L. Incubation was performed in a constant-temperature cabinet at 37°C for 18 h. Cocultivation of bacterial strains was performed in a liquid LB medium in 20-mL test tubes with shaking overnight at 37°C.

Isolation of chromosomal DNA. Fresh biomass was transferred to a microcentrifuge tube and resuspended in 100 μL of a mixture containing 0.15 M NaCl and 0.1 M EDTA (pH 8) (DIA, Germany). Then, the mixture was supplemented with lysozyme (5 mg/mL) and incubated at 37°C for 1 h, after which 20 μL of 20% SDS (AppliChem, Germany) was added and the mixture was incubated at 55–60°C for another 10–15 min. Thereafter, the mixture was supplemented with 60 μL of 5 M NaCl, mixed thoroughly, and centrifuged at 12000 g for 3 min. The supernatant was transferred to a new Eppendorf tube (1.5 mL), extracted with an equal volume of phenol–chloroform–isoamyl alcohol mixture (25 : 24 : 1), and centrifuged at 5000 g. Then, the supernatant was repeatedly extracted under the same conditions and washed three times with an equal volume of chloroform–isoamyl alcohol mixture (24 : 1). After each centrifugation cycle at 5000 g, the supernatant was collected.

Then, the supernatant was mixed with two volumes of 96% ethanol to precipitate DNA, incubated at –20°C for 20 min, and centrifuged at 12 000 g for 3 min. The pellet was washed with 70% ethanol and centrifuged again. The supernatant was carefully removed, and the pellet was dried at 37°C for 20 min and dissolved in 50 μL of water.

PCR conditions. For one experiment, 30 μL of reaction mixture was prepared. Amplification was performed using the following reaction mixtures: 3 μL of PCR buffer, 3 μL of $MgSO_4$ (reagent grade, Khimmed), 3 μL of dNTP (Fermentas, Lithuania), 1 unit of *Taq* polymerase (5 U/μL) (Fermentas, Lithuania), specific primers for PCR (Table 2), 2 μL of the test sample, and deionized water (16.5 μL per sample).

PCR was performed under the following conditions: denaturation at 94°C for 5 min; denaturation at 94°C for 40 s, annealing (for the temperature for each pair of primers, see Table 2) for 30 s; elongation at 72°C for 20 s (30 cycles); and elongation at 72°C for 5 min.

Electrophoretic separation of PCR fragments was performed in a 1% agarose gel (Fermentas, Lithuania) at a voltage of 5 W/cm^3 for 30 min. A once diluted solution of TAE buffer (50 × TAE containing 242 g of Tris base (Pancreac, Spain), 57 mL of glacial acetic acid (reagent grade) (Mosreaktiv, Russia), and 14.6 g EDTA (analytical grade, DiaM) in 1 L (pH 8.0)) was used as an electrophoretic buffer. Agarose (1 g) (Fermentas, Lithuania) in 100 mL of TAE buffer was heated until it completely dissolved. Then, slightly

Table 2. Primers for PCR

Microorganism	Primer sequence	Primer annealing temperature, °C	Amplicon size, bp.
B. subtilis	Bsub-f CAAGAATGTCTAAGATCTCG Bsub-r CAGTCGGGAAATCAGGC	48	268
B. amyloliquefaciens	Bamy-fAAATCTGCCCGTATCGTCGGT Bamy-r GTGAGCATTGGCGTCACGGCG	60	747
B. licheniformis	Blich-fAAGCCGGTGCACAGAAGAATAG Blich-r TCGATTTTCTTATCGCGCACT	51	734
B. atrophaeus	Batr-f CACCCTCACGGAGATTCCGCA Batr-r AATTCTTTCTTTCCCTGATGG	54	541
B. mojavensis	Bmqj-f CGTTATCGTATCCCGGGCA Bmoj-r AAAATTCTTTCTTTCCCTGAC	60	685
B. vallismortis	Bval-f CGGATGTTCGTGACGGTTTAC Bval-r CCGCAGTCGGGAAGTCAGGA	60	538
B. sonorensis	Bson-f CTTGTTCAAGGCCATGGG Bson-r CCAAATGATGTTTGAAGT	57	684

Table 3. Homology between the nucleotide sequences of the 16S rRNA genes of type strains of closely related bacterial species belonging to the *B. subtilis* group

Sequence code in GenBank	Degree of homology	Fragment size, bp	Microorganism, strain
S000003473	0.988	1423	*B. subtilis* (T); DSM10; AJ276351
S000014133	0.991	1426	*B. atrophaeus* (T); JCM9070; AB021181
S000390972	0.998	1347	*B. sonorensis* (T); NRRL B-23154; AF302118
S000417319	0.998	1435	*B. mojavensis* (T); IF015718; AB021191
S000428475	0.988	1337	*B. subtilis* (T); NRRL B-23049; AF074970
S000734915	0.990	1389	*B. amyloliquefaciens* (T); NBRC 15535; AB255669
S000858645	0.998	1463	*B. licheniformis* (T); ATCC 14580; DSM 13; CP000002

chilled agarose was added to the plates with a comb (Bio-Rad, United States) and allowed to congeal. The once diluted TAE solution was loaded on the gel. The test sample colored with the loading buffer (Fermentas, Lithuania) was added to the agarose wells.

RESULTS AND DISCUSSION

Phylogenetic Analysis of Bacteria of the B. subtilis Group

For phylogenetic analysis, we used the following microorganisms: *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. vallismortis, B. atrophaeus, B. sonorensis,* and *B. mojavensis*.

A comparison of the nucleotide sequences of ribosomal RNA genes showed that, in most cases, the degree of homology between them was greater than 99% (Table 3). An analysis was performed using the special RDP10 program [11].

On the basis of these data, a phylogenetic tree of bacteria of the *B. subtilis* group was constructed (Fig. 1).

The criterion for classifying a microorganism to a particular species is a degree of homology of its DNA with the DNA of the strains in this group of at least 97%. Therefore, using this criterion, it is impossible to reliably distinguish between species of the *B. subtilis* group on the basis of structural distinctions between ribosomal RNA genes: any phylogenetic analysis based on the sequence of these genes should be supplemented by a comparison of the composition of other target genes.

Identification of an Additional Target for the Molecular Identification of Closely Related Species of Bacteria Belonging to the B. subtilis Group

The main criterion in selecting a target gene was its primary structure. The nucleotide composition of a target should provide the possibility to reliably distinguish between the seven closely related species of the *B. subtilis* group. It is possible if the nucleotide

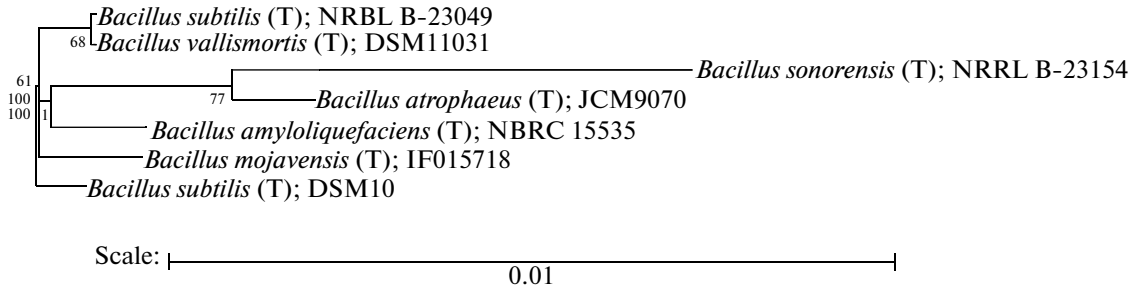


Fig. 1. Phylogenetic tree of bacillus type strains belonging to the *B. subtilis* group according to the 16S rRNA gene sequence.

sequences of the studied target genes of relevant species are homologous by at most 96%.

In papers devoted to the taxonomy of bacilli, genotyping was usually performed using the following operational genes: *rpoB* (encodes RNA polymerase), *secY* (encodes a membrane protein), *gyrB* (encodes the beta subunit of DNA gyrase), and *gyrA* (encodes the alpha subunit of DNA gyrase). However, the methods described in literature do not allow for a rapid diagnosis of all seven closely related species of bacteria belonging to the *B. subtilis* group; moreover, the published methods can only be used if additional sequencing of fragments of the target gene DNA is performed. We analyzed the nucleotide sequences of the *recA, rpoB, secY, gyrA*, and *gyrB* genes of the bacteria of the *B. subtilis* group. It was found that not all sequences of operational genes, such as *recA, rpoB, secY*, and *gyrB*, are known for the seven strains studied.

The criteria for the possibility of using various genes are the knowledge of the respective nucleotide sequence and the degree of its homology with the DNA of the strains within the given group. These criteria are best met by the target *gyrA* gene, which encodes the alpha subunit of DNA gyrase.

To identify the possibility of using the operational *gyrA* gene as a target, we performed an alignment of both full-length and partial nucleotide sequences of these genes for all seven species of bacteria belonging to the group *B. subtilis* that were available in GenBank [12]. This work was complicated by the fact that, for the species *B. vallismortis, B. atrophaeus, B. sonorensis*, and *B. mojavensis*, only partial nucleotide sequences of the *gyrA* genes are available and the sequences of the type strains of these species are absent.

Using the ClustalW Multiple Sequence Alignment program [13], we performed multiple alignment and assessed the homology of a number of nucleotide sequences of the *gyrA* genes (Fig. 2, Table 4). Table 4 shows the sequence similarity of *gyrA* gene fragments of the strains analyzed.

As can be seen from the presented data, the homology of the nucleotide sequences of the *gyrA* genes in bacteria of the *B. subtilis* group varied from 73.1 to 95.6%. This degree of homology allows the *gyrA* gene to be used as a target for selecting species-specific primers.

Selection of Primers for the Species-Specific Identification of Species of the B. subtilis Group

An analysis of the alignment results of the nucleotide sequences of the *gyrA* genes of bacteria belonging to the *B. subtilis* group revealed regions unique to each

Table 4. Percentage of similarity of *gyrA* gene fragments in *B. subtilis* reference strains

No.	Reference strain	*B. atrophaeus*	*B. subtilis*	*B. amyloliquefaciens*	*B. licheniformis*	*B. vallismortis*	*B. mojavensis*	*B. sonorensis*
1	*B. atrophaeus*	100	79.2	79.6	74.4	80.6	95.6	74.3
2	*B. subtilis*			79.5	75.3	92.9	82.2	73.8
3	*B. amyloliquefaciens*				75.1	81.5	82.7	75.9
4	*B. licheniformis*					75.5	72.7	86.4
5	*B. vallismortis*						81.3	73.2
6	*B. mojavensis*							73.1
7	*B. sonorensis*							100.0

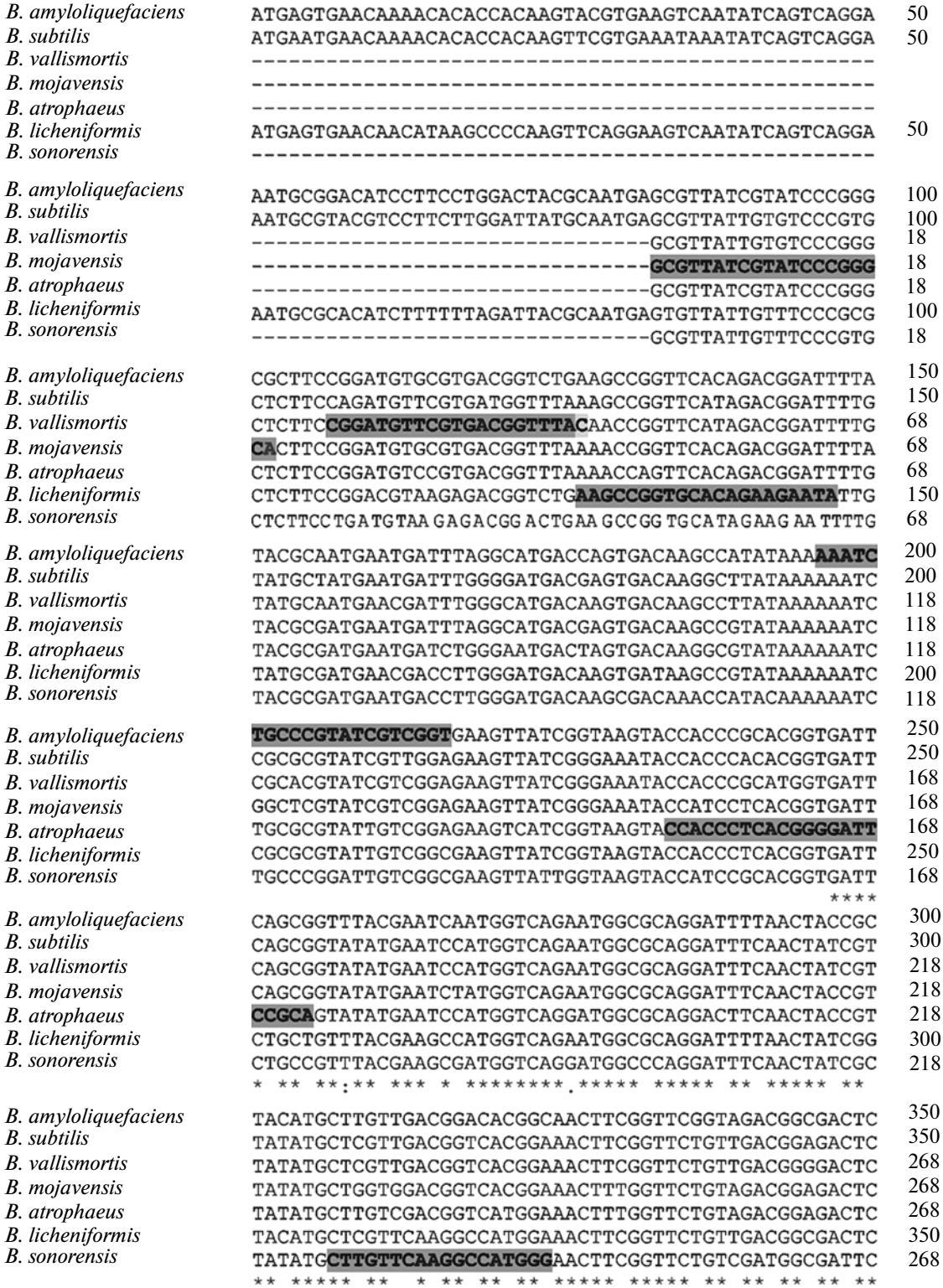
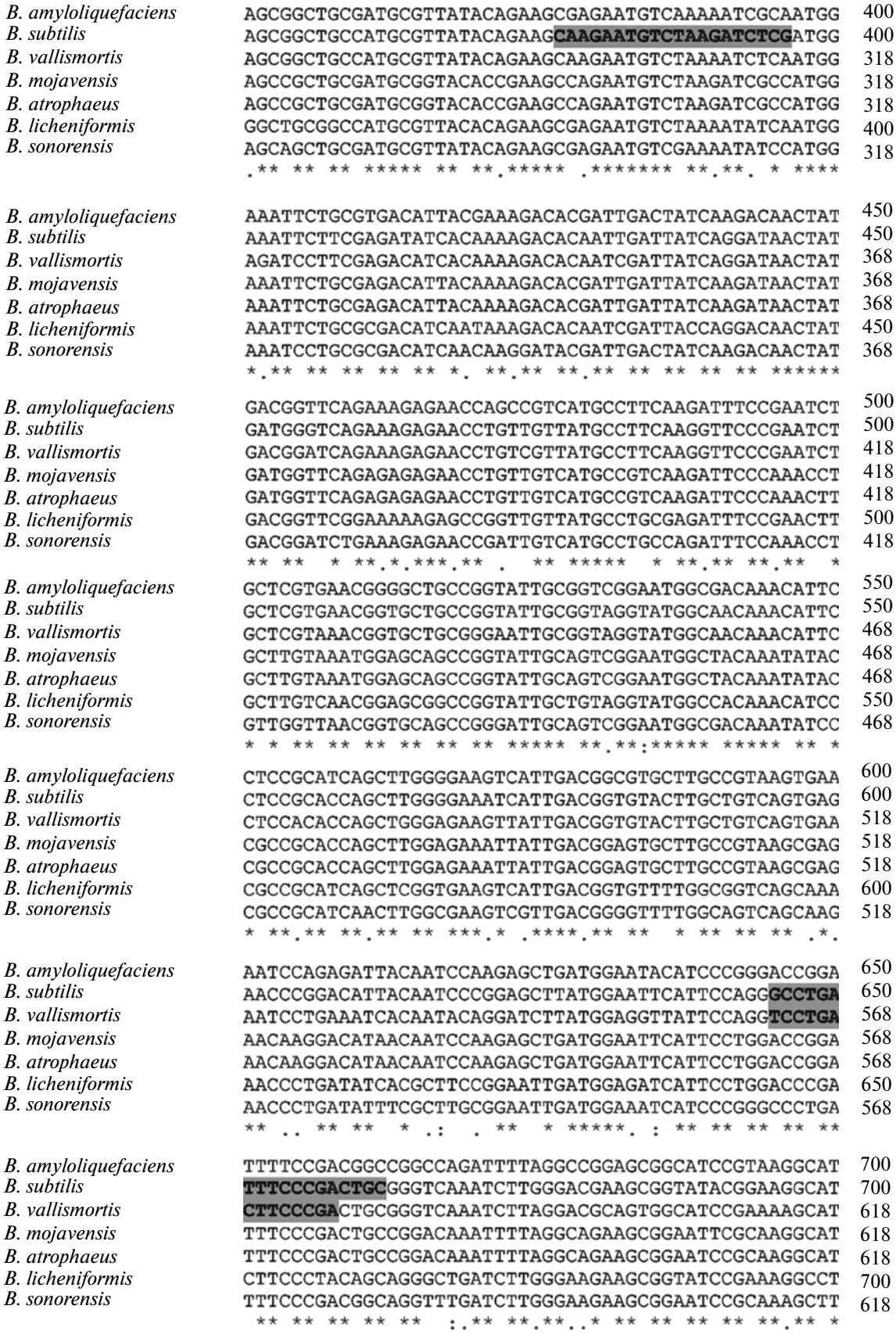


Fig. 2. Alignment of the nucleotide sequences of the *gyrA* genes of the reference strains.

B. subtilis >gi|305672698:7005-9470 *Bacillus subtilis* subsp. *spizizenii* str. W23, complete genome; *B. amyloliquefaciens* > gb|CP002927.1|:7010-9469 *Bacillus amyloliquefaciens* XH7, complete genome; *B. licheniformis* >gb|AE017333.1|:6900-9368 *Bacillus licheniformis* DSM 13, complete genome; *B. vallismortis* >gi|160905565|gb|EU138601.1| *Bacillus vallismortis* strain NRRL B-14890 *gyrA* gene, partial sequence; *B. atrophaeus* >gi|160905665|gb|EU138651.1| *Bacillus atrophaeus* strain NRRL BD-622 *gyrA* gene, partial sequence; *B. mojavensis* >gi| 160905651 |gb|EU138644.11 *Bacillus mojavensis* strain NRRLBD-600 *gyrA* gene, partial sequence; *B. sonorensis* > gi|160905585|gb|EU138611.1| *Bacillus sonorensis* strain NRRL B-23154 *gyrA* gene, partial sequence.

* Full match of sequences; the unique regions (primers) in the sequence of the given species are shown in bold.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B. vallismortis AGCGGCTGCCATGCGTTATACAGAAGCAAGAATGTCTAAAATCTCAATGG 318
B. mojavensis AGCCGCTGCGATGCGGTACACCGAAGCCAGAATGTCTAAGATCGCCATGG 318
B. atrophaeus AGCCGCTGCGATGCGGTACACCGAAGCCAGAATGTCTAAGATCGCCATGG 318
B. licheniformis GGCTGCGGCCATGCGTTACACAGAAGCGAGAATGTCTAAAATATCAATGG 400
B. sonorensis AGCAGCTGCGATGCGTTATACAGAAGCGAGAATGTCGAAAATATCCATGG 318
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B. licheniformis AAATTCTGCGCGACATCAATAAAGACACAATCGATTACCAGGACAACTAT 450
B. sonorensis AAATCCTGCGCGACATCAACAAGGATACGATTGACTATCAAGACAACTAT 368
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B. subtilis GATGGGTCAGAAAGAGAACCTGTTGTTATGCCTTCAAGGTTCCCGAATCT 500
B. vallismortis GACGGATCAGAAAGAGAACCTGTCGTTATGCCTTCAAGGTTCCCGAATCT 418
B. mojavensis GATGGTTCAGAGAGAGAACCTGTTGTCATGCCGTCAAGATTCCCAAACCT 418
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B. licheniformis GACGGTTCGGAAAAAGAGCCGGTTGTTATGCCTGCGAGATTTCCGAACTT 500
B. sonorensis GACGGATCTGAAAGAGAACCGATTGTCATGCCTGCCAGATTTCCAAACCT 418
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B. mojavensis TTTCCCGACTGCCGGACAAATTTTAGGCAGAAGCGGAATTCGCAAGGCAT 618
B. atrophaeus TTTCCCGACTGCCGGACAAATTTTAGGCAGAAGCGGAATCCGCAAGGCAT 618
B. licheniformis CTTCCCTACAGCAGGGCTGATCTTGGGAAGAAGCGGTATCCGAAAGGCCT 700
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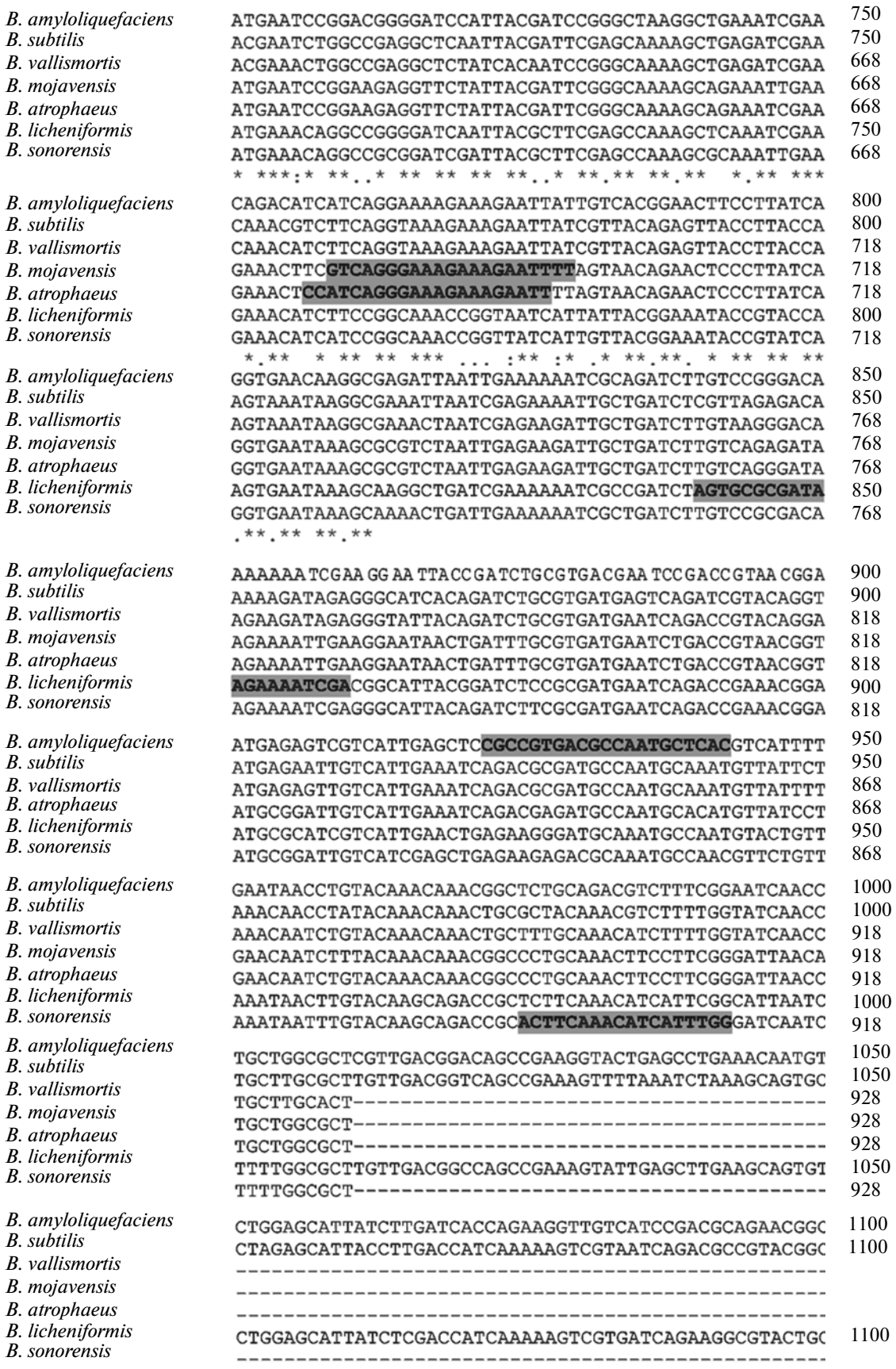
Fig. 2. Contd.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B. licheniformis GAAACATCTTCCGGCAAACCGGTAATCATTATTACGGAAATACCGTACCA 800
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B. subtilis AAACAACCTATACAAACAAACTGCGCTACAAACGTCTTTTGGTATCAACC 1000
B. vallismortis AAACAATCTGTACAAACAAACTGCTTTGCAAACATCTTTTGGTATCAACC 918
B. mojavensis GAACAATCTTTACAAACAAACGGCCCTGCAAACTTCCTTCGGGATTAACA 918
B. atrophaeus GAACAATCTGTACAAACAAACGGCCCTGCAAACTTCCTTCGGGATTAACC 918
B. licheniformis AAATAACTTGTACAAGCAGACCGCTCTTCAAACATCATTCGGCATTAATC 1000
B. sonorensis AAATAATTTGTACAAGCAGACCGCACTTCAAACATCATTTGGGATCAATC 918
B. amyloliquefaciens TGCTGGCGCTCGTTGACGGACAGCCGAAGGTACTGAGCCTGAAACAATGT 1050
B. subtilis TGCTTGCGCTTGTTGACGGTCAGCCGAAAGTTTTAAATCTAAAGCAGTGC 1050
B. vallismortis TGCTTGCACT---------- 928
B. mojavensis TGCTGGCGCT---------- 928
B. atrophaeus TGCTGGCGCT---------- 928
B. licheniformis TTTTGGCGCTTGTTGACGGCCAGCCGAAAGTATTGAGCTTGAAGCAGTGT 1050
B. sonorensis TTTTGGCGCT---------- 928
B. amyloliquefaciens CTGGAGCATTATCTTGATCACCAGAAGGTTGTCATCCGACGCAGAACGGC 1100
B. subtilis CTAGAGCATTACCTTGACCATCAAAAAGTCGTAATCAGACGCCGTACGGC 1100
B. vallismortis ----------
B. mojavensis ----------
B. atrophaeus ----------
B. licheniformis CTGGAGCATTATCTCGACCATCAAAAAGTCGTGATCAGAAGGCGTACTGC 1100
B. sonorensis ----------

Fig. 2. Contd.

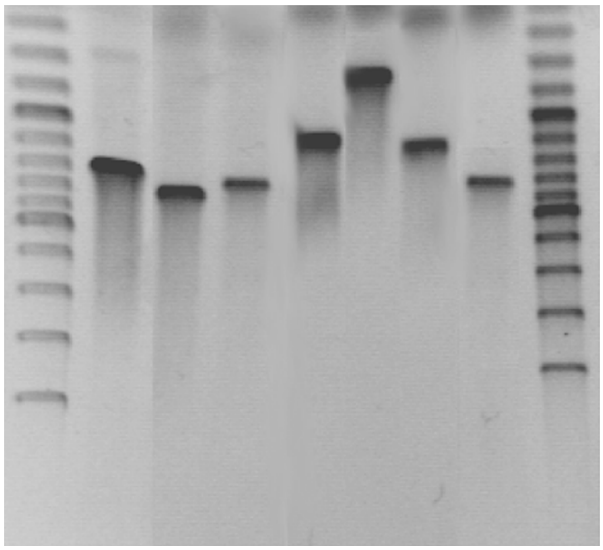

Fig. 3. Electrophoretogram of DNA amplification products obtained with the use of bacillus type strains of the *B. subtilis* group: (*1, 9*) DNA length markers (O'GeneRuler 100 bp DNA Ladder; 100, 200, 300,400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, and 3000 bp, from top to bottom); (*2*) *B. sonorensis* DSM 13779 and NRRL B-23154 B-11122, primers Bson-f and Bson-r; (*3*) *B. amyloliquefaciens* DSM 7 and ATCC 23350 B-9866, primers Bamy-f and Bamy-r; (*4*) *B. mojavensis* DSM 9205, ATCC 51516, and NRRL B-14698 B-11119, primers Bmoj-f and Bmoj-r; (*5*) *B. vallismortis* DSM 11031 and NRRL B-14890 B-11123, primers Bval-f and Bval-r; (*6*) *B. subtilis* DSM 10 and ATCC 6051 B-9865, primers Bsub-f and Bsub-r; (*7*) *B. atrophaeus* DSM 7264, ATCC 49337, and NRRL-NRS 213 B-11116, primers Batr-f and Batr-r; and (*8*) *B. licheniformis* ATCC 14580, DSM 13, NCIB 9375, NCTC 10341, and NRRL NRS-1264 B-10956, primers Blich-f and Blich-r.

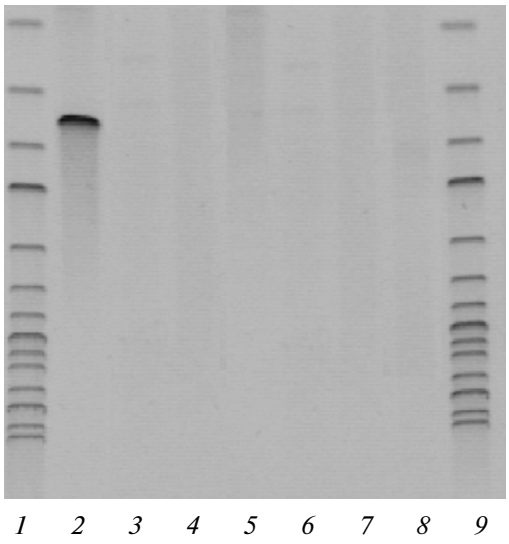

Fig. 4. Electrophoretogram of DNA amplification products obtained with the use of strain B-1517: (*1, 9*) DNA length markers 1 kb DNA Gene-Ruler (10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250 bp, from bottom up); (*2*) primers Bson-f and Bson-r; (*3*) primers Bamy-f and Bamy-r; (*4*) primers Bmoj-f and Bmoj-r; (*5*) primers Bval-f and Bval-r; (*6*) primers Bsub-f and Bsub-r; (*7*) primers Batr-f and Batr-r; and (*8*) primers Blich-f and Blich-r.

species (Fig. 2, shown in bold), which are important for selecting species-specific primers.

The pairs of primers used for the species-specific identification of bacteria (Table 2) were selected using the Oligos v. 9.0 software.

Selection of Components for Species-Specific PCR Systems and Optimization of the Reaction Conditions

To optimize the conditions for the species-specific determination of a group of closely related species of bacilli *B. subtilis* using primers selected on the basis of the nucleotide sequences of the *gyrA* genes, typical strains of bacteria are used in test species (see Table 1.).

The conditions for PCR reactions were determined using the Oligos v. 9.0 software. The chromosomal DNA isolated from pure cultures of type strains of the bacilli examined was used as a template.

For each pair of species-specific primers, we selected such PCR conditions that ensured the stable formation of amplicons of the expected size and minimized the formation possibility of nonspecific fragments (Table 2). An electrophoretogram of DNA amplification products with species-specific primers using the chromosomal DNA of *Bacillus* type strains as a template is shown in Fig. 3.

The data presented in Fig. 3 show that, in all cases, amplicons of the expected size were obtained: 684 bp for primers Bson-f and Bson-r; 538 bp for primers Bval-f and Bval-r; 695 bp for primers Bmoj-f and Bmoj-r; 541 bp for primers Batr-f and Batr-r; 734 bp for primers Blich-f and Blich-r; 747 bp for primers Bamy-f and Bamy-r, and 268 bp for primers Bsub-f and Bsub-r.

When PCR amplification was performed under optimal conditions, there was not a single false positive cross-reaction.

Thus, specific PCR systems based on the *gyrA* genes were designed and elaborated using the type strains of bacilli of the *B. subtilis* group: *B. sonorensis, V. vallismortis, B. mojavensis, B. atrophaeus, B. licheniformis, B. amyloliquefaciens*, and *B. subtilis.*

Examples of Using the Developed Technique for the Species Identification of Collection Strains

Using the method developed, we identified VKPM strains B-1517, B-2984, and B-1283. Previously, all three strains were identified on the basis of the 16S RNA gene sequence. It was found that all three strains belong to the *B. subtilis* group; however, it was impos-

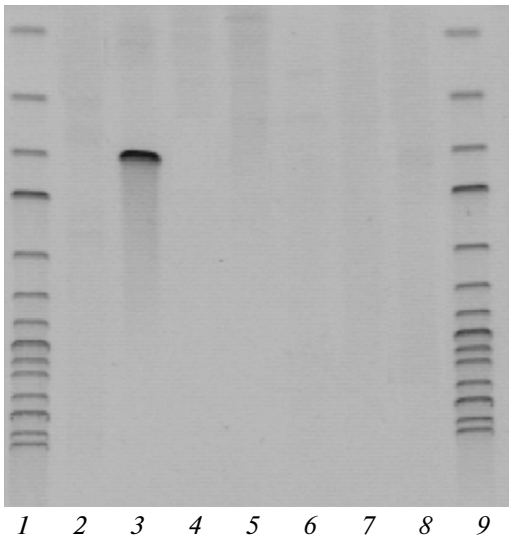

Fig. 5. Electrophoretogram of DNA amplification products obtained with the use of strain B-2984: (*1*, *9*) DNA length markers 1kb DNA GeneRuler (10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500,1000, 750, 500, and 250 bp, from bottom up); (*2*) primers Bson-f and Bson-r; (*3*) primers Bamy-f and Bamy-r; (*4*) primers Bmoj-f and Bmoj-r; (*5*) primers Bval-f and Bval-r; (*6*) primers Bsub-f and Bsub-r; (*7*) primers Batr-f and Batr-r; and (*8*) primers Blich-f and Blich-r.

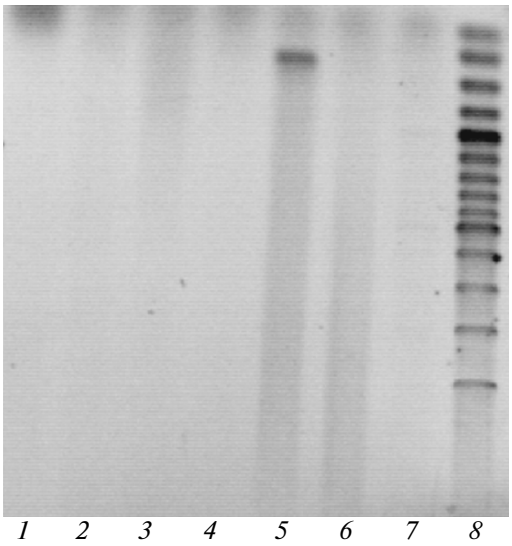

Fig. 6. Electrophoretogram of DNA amplification products obtained with the use of strain B-1283: (*1*) primers Bson-f and Bson-r; (*2*) primers Bamy-f and Bamy-r; (*3*) primers Bmoj-f and Bmoj-r; (*4*) primers Bval-f and Bval-r; (*5*) primers Bsub-f and Bsub-r; (*6*) primers Batr-f and Batr-r; (*7*) primers Blich-f and Blich-r; and (*8*) DNA length markers O'GeneRuler 100 bp DNA Ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, and 3000 bp, from top to bottom).

sible to reliably assign them to a certain species of bacteria in this group.

The PCR results with the use of species-specific primers based on the *gyrA* gene are shown in Figs. 4–6.

As can be seen in Fig. 4, a positive result (684-bp amplicon) was obtained with the use of primers Bson-f and Bson-r specific to *B. sonorensis* , whereas amplification with the use of other primers specific to the other six species of bacilli yielded a negative result. Thus, the B-1517 strain can be assigned to the species *B. sonorensis*.

It can be seen in Fig. 5 that a positive result (747-bp amplicon) was obtained using primers Bamy-f and Bamy-r specific to *B. amyloliquefaciens*, whereas amplification with the use of other primers specific to the other six species of bacilli gave a negative result. Thus, on the basis of these data, strain 2984 was assigned to the species *B. amyloliquefaciens*.

Figure 6 shows that a positive result (268-bp amplicon) was produced using primers Bsub-f and Bsub-r specific to *B. subtilis*, whereas amplification with the use of other primers gave a negative result. Thus, strain 1283 was assigned to the species *B. subtilis*.

Thus, species-specific PCR systems based on the *gyrA* gene sequence can be used as auxiliary tools for the precise identification of bacilli belonging to the *B. subtilis* group in addition to identification by the nucleotide composition of the ribosomal genes.

Application of the Developed Method for the Species Identification of Bacillus Strains Belonging to the B. subtilis group in Mixed Cultures

To test the possibility of the detection and identification of bacilli of the *B. subtilis* group in mixed cultures and microbial preparations, we performed the following model experiment. Collection strains VKPM B-1517, B-2984, and B-1283 were grown together, after which total DNA was isolated, which was used as a template in PCR with the use of primers based on the *gyrA* gene sequence specific for each of the seven *Bacillus* species of the *B. subtilis* group (Fig. 7).

The data presented in Fig. 7 show that relevant amplicons were found in three cases: 684-bp amplicon with primers Bson-f and Bson-r; 747-bp amplicon with primers Bamy-f and Bamy-r, and 268-bp amplicon with primers Bsub-f and Bsub-r. With the use of the remaining four pairs of primers, amplicons were not obtained. Thus, using species-specific primers, we established that the bacterial mixture, the total DNA of which was used for PCR, included *B. sonorensis, B. subtilis*, and *B. amyloliquefaciens*.

The results of the model experiment allow us to postulate that the identification method of bacilli of the *B. subtilis* group developed in this study can be used to detect strains belonging to this group of species in complex bacterial preparations without an obligatory microbiological separation of strains.

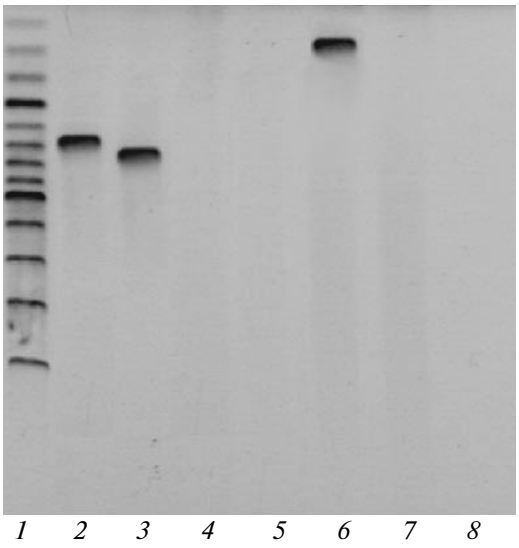

Fig. 7. Electrophoretogram of DNA amplification products obtained using a mixture of strains B-1517, B-2984, and B-1283: (*1*) DNA length markers O'GeneRuler 100 bp DNA Ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, and 3000 bp, from top to bottom); (*2*) primers Bson-f and Bson-r; (*3*) primers Bamy-f and Bamy-r; (*4*) primers Bmoj-f and Bmoj-r; (*5*) primers Bval-f and Bval-r; (*6*) primers Bsub-f and Bsub-r; (*7*) primers Batr-f and Batr-r; and (*8*) primers Blich-f and Blich-r.

Thus, the results of this study show the possibility of using a technique based on the PCR analysis of the *gyrA* gene variation for the species-specific determination of closely related *Bacillus* strains without cloning and sequencing, which results in saving time and material resources. In addition, the developed approach can be used to determine the species composition of bacterial strains contained in complex probiotic preparations without their preliminary microbiological separation.

The efficiency of the developed technique for the species *B. subtilis, B. amyloliquefaciens*, and *B. licheniformis* was confirmed not only by the examples in this paper but also by its successful use for the species identification of more than 30 VKPM strains (data not shown).

The assumption regarding the possibility of using this method for the identification of the *B. sonorensis, B. vallismortis, B. mojavensis*, and *B. atrophaeus* species is still based on a limited number of examples. For a more definitive conclusion regarding the effectiveness of this technique for the listed species, further accumulation of statistics is required.

ACKNOWLEDGMENTS

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