IDENTIFICATION OF INDUSTRIALLY IMPORTANT LACTIC ACID BACTERIA IN FOODSTUFFS

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Abstract: Universal genus-specific primers for comparative analysis of two aligned 16S rRNA gene nucleotide sequences of lactic acid bacteria were constructed. The method to identify lactic acid bacteria and a comprehensive plan for their genus and species identification may be used to characterize isolated strains of the *Lactobacillus* genus bacteria and in quality control of foodstuffs enriched with *Lactobacillus*.

Key words: lactic acid bacteria, primers, nucleotide sequence, 16S rRNA gene, identification, PCR, phylogenetic analysis, construction

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INTRODUCTION

Strains of the *Lactobacillus* genus, most subspecies of which transform lactose and other carbohydrates into lactic acid, are industrially important lactic acid bacteria.

This genus of microorganisms is most frequently used in production of foodstuffs of animal and plant origin by fermentation. The *Lactobacillus* genus is divided into three subspecies by the niche they occupy and their ability to ferment substances, for example, *L. delbruekcii* subsp. *delbrueckii* are usually found in vegetables treated with enzymes while *L. delbrueckii* subsp. *bulgaris* and *L. delbrueckii* subsp. *lactis*, are typically present in dairy products with addition of wide variety of carbohydrates. Specificity of industrial characteristics and vital processes makes strains of the *Lactobacillus* subspecies convenient and widely used in industry both individually and in consortium with other species to produce lactic acid dietary products, including cheese and yogurt [1, 3].

Simplicity and reliability of *L. delbrueckii* identification at various levels, that is level of strain and level of subspecies, makes them interesting not only for fundamental knowledge but for practical applications as well. Identification of microorganisms indicated on the label of a product by the manufacturer should correspond to those microorganisms that are used at a particular plant to that are applied for obtain cheese and various kinds of fermented milk products in reality.

Today, application of taxonometric identification of *L. delbrueckii* becomes urgent since there is a chance of mistake in case of utilization of phenotypic methods. These methods are based on utilization of genetic methods using strain-specific oligonucleotide probes upon blot-hybridization, DNA fingerprinting, or ribosomal DNA restriction. Today, precise and rapid identification of lactic acid bacteria is possible owing to development of methods based on polymerase chain reaction (PCR) [2, 3].

Therefore, in case rapid result is needed, methods based on PCR will be considered an alternative to microbiology tests and find wide application. Application of PCR methods allows for high-precision identification and determination of genus and species.

In the process of construction of genus- and species-specific primers, typically, 16S or 23S rRNA gene fragments, specific for each species and genus, and hypervariable internal transcribed regions (ITS), separating the above-mentioned loci, are chosen [4]. Also, transaldolase gene, small recA protein gene involved in recombination of homologous DNA, or tuf-gene coding for Tu elongation factor may serve as templates [5, 6].

In various species of *Lactobacillus*, the 16S rRNA genetic determinant has approximately the same size of about 1500 bp and is present in bacterial genome in several copies. Spacer regions of various size serve as separators between the copies of bacterial genes. Presence of two flanked spacer regions different in size between 16 and 23S rRNA genes is typical of the *Lactobacillus* genus. The 16S rRNA gene, containing both variable and conservative regions in the nucleotide sequence, is the most appropriate for determination of genus and species affiliation of a bacterium.

Therefore, the aim of the work was to construct genus- and species-specific primers to detect and identify industrially important strains of the *Lactobacillus* genus.

MATERIALS AND METHODS

In the work we used ten bacterial strains of the *Lactobacillus* genus registered in the All-Russian Collection of Industrial Microorganisms (VKPM; FGUP GosNII-genetika), five of which, according to the passport data, belong to the *L. acidophilus* species, and the other five, to *L. delbruesckii* ssp. *bulgaricus* (Table 1), and 12 nucleotide sequences of 16S rRNA genes of lactic acid bacteria deposited in the NCBI data base (Table 2).

Table 1. Microorganisms of the All-Russian Collection of Industrial Microorganisms (VKPM; FGUP GosNII-genetika)

No.	VKPM ID	Strain title	Source
1	V-6551	L.a. T-3	Feces of a newborn
2	V-8153	L.a. AE-5	Obtained from the L.a. AT-44 strain
3	V-194	L.a.1k	Isolated from chicken
4	V-8634	L.a. 3	Self-fermented curdled milk
5	V-5863	L.a. 57S	Self-fermented milk
6	V-6516	L.d. b. 21	Isolated from milk
7	V-3964	L.d. b.	Raw milk
8	V-3141	L.d. b. L20/2	Matzoon
9	V-6543	L.d. b. B-259	Self-fermented milk product
10	V-6515	L.d. b. 19	Isolated from milk

Table 2. Record numbers of 16S rRNA gene sequences of the *Lactobacillus* genus representatives deposited in NCBI database

Record number of 16S rRNA gene sequence in NCBI	Strain title
CP000033.3	*Lactobacillus acidophilus* NCFM
FJ556999.1	*Lactobacillus acidophilus* strain CECT 4529
FJ749655.1	*Lactobacillus acidophilus* strain IMAU30067
EU878007.1	*Lactobacillus acidophilus* strain NX2-6
AY763429.1	*Lactobacillus acidophilus* strain LH4
FJ861093.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain KLDS 1.0625
EU483107.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain LC
CP000412.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365
EU642554.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU40169
FJ915706.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU40169
EU547306.1	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain BCS113
AF429503	*Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842

Isolation of DNA. DNA from dry bacteria culture was isolated using the Kit for Isolation of Genomic DNA from Bacteria (Sintol, Moscow). To extract bacterial DNA, cells were sedimented from 1 mL liquid culture by centrifugation at 10000 rpm for 3 min and the supernatant was removed. Then, cells were resuspended in 300 μL buffer (10 mM Tris HCl, pH 8.0; 50 mM glucose, 10 mM EDTA) and 3 μL lysozyme (10 mg/mL) was added to the suspension. Cell wall was lysed at 37°C for 60 min under occasional stirring of the tube content by overturning it, then the suspension was centrifuged at 13000 rpm for 1 min. The sediment was resuspended in 300 μL of lysing buffer (20 mM Tris-HCl, pH 8.0; 75 mM NaCl; 1% SDS; 10 mM EDTA) and 3 μL RNase A (10 mg/mL) was added to the mixture. Incubation was performed at 37°C for 30 min, then the mixture was cooled on ice for 1 min. Then, 100 μL solution for protein precipitation (7.5 M ammonium acetate) was added to the mixture and mixed on a vortex for 20 s, then it was centrifuged at 13000 rpm for 5 min. The supernatant was transferred into clean 1.5 mL tubes and 300 μL isopropanol were added, the mixture was mixed by overturning for 1 min and placed at –20°C for 30 min. The mixture was centrifuged at 13000 rpm for 5 min, the supernatant was accurately decanted, and the tubed were placed overturned on a clean filter. Then, 400 μL of 70% ethanol was added and mixed several times by overturning to wash the DNA sediment. The procedure was repeated several times. Finally, the sediment was dried at 37°C 15 min till ethanol drops disappeared completely. The dried sediment was dissolved in 30 μL TE buffer.

Phylogenetic analysis. To construct species-specific and genus-specific primers, 12 nucleotide sequences of 16S rRNA gene from lactic acid bacteria deposited in the NCBI database were used: *Lactobacillus acidophilus* CP000033.3, FJ556999.1, FJ749655.1, EU878007.1, and AY763429.1; *Lactobacillus delbruesckii* ssp. *bulgaricus* FJ861093.1, EU483107.1, CP000412.1, EU642554.1, FJ915706.1, and EU547306.1 (for species-specific primers); *Lactobacillus delbruesckii* ssp. *bulgaricus* AF429503 and *Lactobacillus acidophilus* AY763429 (for genus-specific primers).

Analysis of DNA sequence coding for 16S rRNA synthesis is the basis for phylogenetic analysis of bacteria. To determined differences in sequences, comparative analysis and construction of phylogenetic tree were performed in ClustalW software.

Oligonucleotide synthesis was performed on an ASM1000 (Biosset, Novosibirsk) DNA/RNA synthesizer. Purification was performed in a polyacrylamide gel.

Amplification of 16S rRNA gene was performed on a Tertsyk equipment (Moscow) using a thermostable Taq polymerase (SibEnzim, Nosvosibirsk) according to the manufacturer's recommendations. The synthesized primers were used for amplification.

Temperature–time profile of PCR was the following: 95°C for 200 s, one cycle; 62°C for 50 s and 95°C for 20 s, 25 cycles; and 72°C for 120 s, one cycle. Analysis of PCR products was performed using electrophoresis in 1.5% gel, containing ethidium bromide. The results were documented using a Vitran (Biokom) videosystem. DNA fragment length marker was used.

RESULTS AND DISCUSSION

To construct universal genus-specific primers, comparative analysis of two aligned nucleotide sequences of 16S rRNA genes from lactic acid bacteria was performed. Results of the analysis allowed for construction of universal primers for detection of the *Lactobacillus* genus bacteria on the basis of 16S rRNA gene,

16S for: 5'- AGA GTT TGA TCC TGG CTC AGG A
and
16S rev: 5'- ACG CTT GCC ACC TAC GTA TTA C,

for amplification of 566 bp-long 16S rRNA gene sequence.

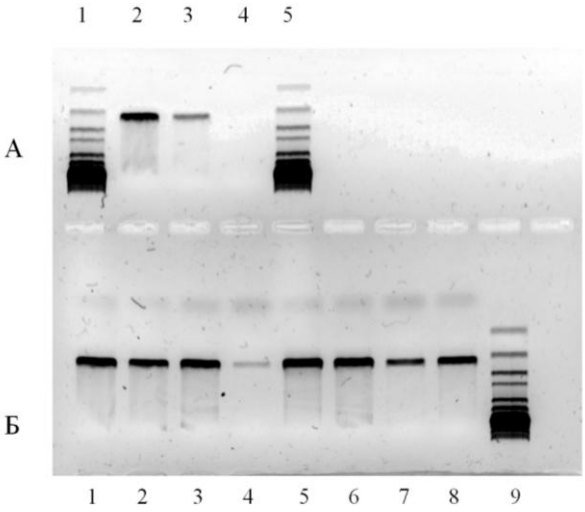

Fig. 1. Electrophoregram of lactic acid bacteria 16S rRNA gene fragment amplification products using synthetic primers: (A) *1*, Medigen 400–2500 bp weight marker, *2*, no. 9, *3*, no. 10, *4*, negative control (H_2O), *5*, marker; (B) *1*, no.1, *2*, no. 2, *3*, no. 3, *4*, no. 4, *5*, no. 5, *6*, no. 6, *7*, no.7, *8*, no. 8, and *9*, marker.

Theoretical specificity of genus-specific primers was confirmed experimentally in ten strains, five of them belonging to *L. acidophilus* species, and another five, to *L. delbrueckii* ssp. *bulgaricus*. Synthesized primers were used in amplification of the 16S rRNA gene fragment in strains under study (Fig. 1).

The oligonucleotides may form basis for a test system allowing for *Lactobacillus* bacteria detection.

Synthesis of primers for amplification of 16S fragments of lactobacilli allowed for elaboration of a fragment, the size of which is sufficient for genomic characteristics elucidation.

To construct species-specific primers, comparative analysis of 11 complete nucleotide sequences of 16S rRNA fragments of lactic acid bacteria was performed using a ClustalW software.

For this purpose, we searched the database of complete nucleotide sequences corresponding to 16S rRNA genes of reference bacteria strains of the *Lactobacillus* genus and chose *Lactobacillus acidophilus* sequences deposited in NCBI database under record numbers CP000033.3, FJ556999.1, FJ749655.1, EU878007.1, and AY763429.1, and *Lactobacillus delbruesckii* ssp. *bulgaricus* sequences FJ861093.1, EU483107.1, CP000412.1, EU642554.1, FJ915706.1, and EU547306.1.

The chosen nucleotide sequences were used for comparative phylogenetic analysis, the results of which are presented in Fig. 2.

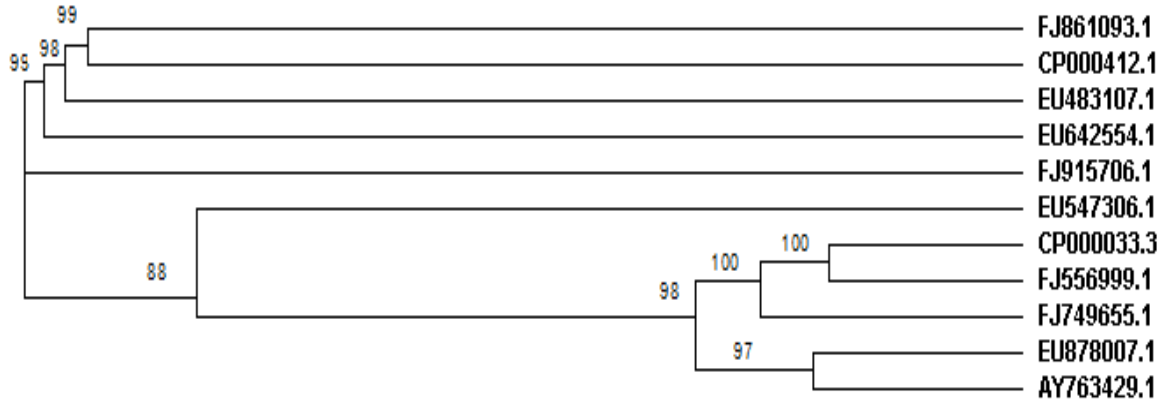

Fig. 2. Phylogenetic tree built based on analysis of 16S rRNA fragment sequences reflecting relationships between lactic acid bacteria *L. bulgaricus* and *L. acidophilus* under study.

The eleven 16S rRNA gene sequences of various lactobacilli that we used were grouped into four clusters: the first cluster contained type strains of the *L. acidophilus* species (CP000033.3, FJ556999.1, FJ749655.1, EU878007.1, and AY763429.1); the second and the third, strains of the *L. bulgaricus* species (EU547306.1 and FJ915706.1, respectively); and the fourth one, strains of the *Lactobacillus delbruesckii* ssp. *bulgaricus* species (FJ861093.1, EU483107.1, CP000412.1, and EU642554.1). The phylogenetic tree (Fig. 1) was built using the ClustalW software. As follows from the data presented in Figure 1, in the first and the fourth clusters two closely related species, *L. acidophilus* and *Lactobacillus delbruesckii* ssp. *bulgaricus*, are joined. According to the data obtained upon the analysis of complete nucleotide sequences of 16S rRNA gene fragments, homology of gene structure between different representatives of the *Lactobacillus* genus with respect to 16S rRNA gene of *Lactobacillus delbruesckii* ssp. *bulgaricus* is 87–93% for the *L. acidophilus* species and 92–99%, for the *L. bulgaricus* strains.

Comparative analysis demonstrated that the most important differences in sequences coding for 16S rRNA are observed in the first third of the sequence. Earlier, foreign authors have systematized species of the *Lactobacillus* genus according to their 16S rRNA genes and other marker sequences.

We optimized the primer sequences proposed previously in order to increase the temperature of annealing and improve specificity of the obtained PCR product. As a result, the following species-specific primers were synthesized,

16SbulF: 5'- CAA CAG AAT CGC ATG ATT CAA GTT TG (26) and

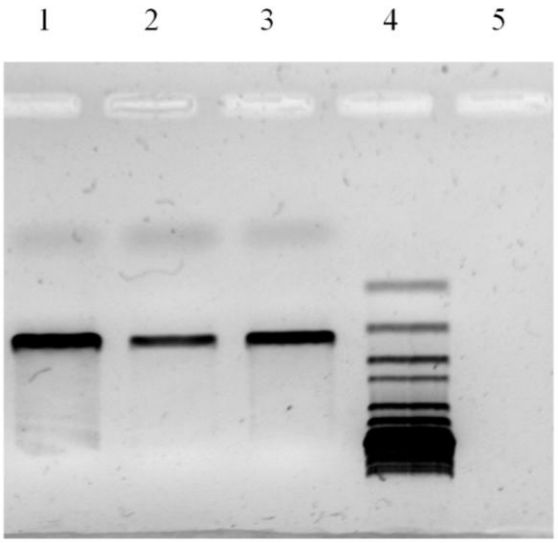

Fig. 3. Electrophoregram of amplification products of genome fragments from different representatives of the *Lactobacillus* genus contained in various substrates: *1*, yogurt; *2*, Bifilaif; *3*, positive control; *4*, molecular weight marker; *5*, negative control.

16SbulR: 5'- ACC GGA AAG TCC CCA ACA CCT A (22),

for amplification of 675 bp-long 16S rRNA sequences.

The newly developed primers possess low homology to the corresponding DNA regions of other lactic acid bacteria, which provides for their specificity. Theoretical specificity of the primers was confirmed experimentally in probiotic products (Fig. 3).

In frames of the research topic, the step of species identification is performed using isolated fragments, which allows for rapid detection of bacteria of the *Lactobacillus* genus in a sample and identification of the *L. acidophilus* and *L. delbruesckii* ssp. *bulgaricus* species. The latter ones are contained in probiotics and sanative foodstuffs and are industrially important. The developed method of detection of lactic acid bacteria and the comprehensive plan for their identification may be used for characteristics of isolated strains of the *Lactobacillus* genus and is of practical interest for applications in quality control of foodstuffs enriched with lactobacilli.

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REFERENCES

1. Tochilina, A.G., Indikatsyya i identifikatsyya bakterii roda lactobacillus s ispol'zovaniem polimeraznoi tsepnoi reaktsii (Detection and identification of bacteria of the Lactobacillus genus using polymerase chain reaction), *Mikrobiologiya, epidemiologiya i immunobiologiya (Microbiology, Epidemiology, and Immunobiology)*, 2008, no. 3, pp. 69–73.
2. Botina, S.G., Identifikatsyya promyshlennykh shtammov molochnokislykh bakterii metodami molekulyarno-geneticheskogo tipirovaniya (Identification of industrial strains of lactic acid bacteria by methods of molecular genetics typing), *Genetika (Genetics)*, 2006, vol. 42, no. 12, pp. 1621–1635.
3. Torriani, S., Zapparoli, F., and Dellaglio, F., Use of PCR-Based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, *Appl. Environ. Microbiol.*, 1999, vol. 65, no. 10, pp. 4351–4356.
4. Heilig, H.G., Zoetendal, E.G., Marteau, P, et al., Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA, *Appl. Environ. Microbiol.*, 2002, vol. 68, no. 1, pp. 114–123.
5. Torriani, S., Felis, G.E., Dellaglio, F., Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA g*ene sequence analysis and multiplex PCR assay with *recA g*ene-derived primers, *Appl. Environ. Microbiol.*, 2001, vol. 67, no. 8, pp. 3450–3454.
6. Ventura, M., Canchaya, C., Meylan, V., et al., Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification, *Appl. Environ. Microbiol.*, 2003, vol. 69, no. 11, pp. 6908–6922.