

MOLECULAR - GENETIC IDENTIFICATION OF LACTIC ACID BACTERIA ON THE BASIS OF THE ANALYSIS OF THE NUCLEOTIDE SEQUENCES OF 16S rRNA GENE

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Abstract

Molecular-genetic analysis of the nucleotide sequence of the 16S rRNA gene of lactic acid bacteria isolated from the gastrointestinal tract healthy people. Identified to species two cultures bacteria of the genus Lactobacillus, which are antagonists against pathogenic and conditionally pathogenic bacteria. By results of the analysis of the nucleotide sequence of the 16S rRNA gene two strains are classified in the genus Lactobacillus. Phylogenetic analysis of the fragment of the 16S rRNA gene showed that strains Lactobacillus plantarum. №22 and Lactobacillus cellobiosus №20 form one cluster with strains of Lactobacillus farraginis and Lactobacillus pentosus. Phylogenetic analysis of the fragment of the 16S rRNA gene showed that the two strains belong to phylogenetic group Lactobacillus buchneri and Lactobacillus plantarum.

Key words: *Lactobacillus, genetic identification, 16S rRNA gene, primers, sequencing, phylogeny*

INTRODUCTION

Microorganisms of the genus Lactobacillus is widely distributed in nature, and some species are the most important representatives of the microbiota of the human body [Bondarenko V., 2003]. Lactobacilli for a long time attracted the attention of scientists - biochemists, microbiologists, physicians, ecologists, because of their potential significance for the maintenance of homeostasis system "man-environment", maintaining health, prevention and treatment of many diseases of different etiologies. Important issue is to obtain new knowledge about the biological properties and molecular genetic structure of lactobacilli; the creation of new probiotic products based on them using different methodological approaches to the cultivation [Tochilina A., 2009].

The main end product of metabolism of lactobacilli is D- and L-lactic acid. In homofermentative lactobacilli lactate is 90% of the fermentation products. Representatives heterofermentative species as well as the final products produced lactic acid and carbon dioxide [Shlegel G., 1987]. Due to the production of organic acids and bacteriocins peroxides many strains of lactobacilli exhibit pronounced antagonistic activity against pathogenic microorganisms [Quadri L., 2002]. Biochemical and morphological properties of lactobacilli are currently the main and sole criterion intergeneric and species identification of microorganisms. However, when only the identification of lactobacilli using standard microbiological tests can be difficult. For example, the identification of closely related species based on physiological and biochemical properties is difficult due to the generality of properties [Botin S., 2008]. Identification of lactic acid bacteria only on the basis of morphological, cultural, physiological and biochemical characteristics in the present is insufficient, because under the influence of various factors, many species have a high level of phenotypic variation [Claesson M., 2007].

For accurate identification of bacteria are currently not sufficient to rely upon data obtained using a method as discriminatory methods have their limitations.

For example, when the phenotypic identification of lactobacilli based on fermentation of carbohydrates researchers often faced with the problem of variations in results depending on the inoculum size, incubation temperature, length of incubation period, the medium composition, surface-to-volume environment, and therefore results obtained by one laboratory often fail correspond to the results of other laboratories can be quite reproducible [Noel R., 2005]. Particularly difficult in phenotypic identification of lactobacilli as Lactobacillus species and strains show a high level of inherent phenotypic variability as evidenced by the results obtained.

When genetic identification on the basis of nucleotide sequence analysis of 16S rRNA gene researchers are faced with the problem of inability to identify genetically related species.

Officially recognized that 97% identity in the nucleotide sequence of 16S rRNA gene is a threshold specific characteristics [Stackebrandt E., 1994]. However, identity among different species sometimes less than 0,5% [Palys T., 1994]. Molecular genetic identification methods have proven to be reliable and independent of external factors [Zeigler D., 2003]. The aim of this work was to identify genetically closely related species of lactobacilli isolated from the gastrointestinal tract of healthy people, using nucleotide sequence analysis of the gene 16S rRNA.

MATERIALS AND RESEARCH METHODS

Bacterial strains and cultivation techniques. The objects of study were lactic bacteria of the genus *Lactobacillus*, registered in the Collection of Industrial Microorganisms "of the Institute of Microbiology and Virology" and passport data belonging to *Lactobacillus plantarum* №22 and *Lactobacillus cellobiosus* №20. Cultures were grown in a medium MRS (Hi-Media, India), for 2 days at 350C.

Medium composition MRS:

1. Peptone 10.0 g
2. Beef extract 10.0 g
3. Yeast extract 5.0 g
4. Dextrose 20.0 g
5. Tween 80 1.0 ml
6. Ammonium citrate 2.0 g
7. Sodium acetate 5.0 g
8. Manganese sulphate 0.05 g
9. Magnesium sulfate 0.1 g
10. Potassium phosphate dibasic 2.0 g
11. Water - 1 liter

Identification and analysis of the nucleotide sequences of the genes 16SrRNA.

Genomic DNA was isolated by Kate Wilson [Wilson K., 1987]. DNA concentration was measured spectrophotometrically using a spectrophotometer Nano Drop 1000 at a wavelength of 260 nm, as well as conducted a qualitative assessment of DNA electrophoretic method.

Matrices for sequencing were synthesized by PCR using universal primers 8f-5'-AgAgTTTgATCCTggCTCag-3 and 806R-5'- ggACTACCAgggTATCTAAT-3 [Vegas E., 2006], allowing to amplify 16S rRNA gene almost completely. The reaction mixture (30 ul) contained 3 l 10x reaction buffer (Fermentas), 2,5 mM MgCl₂, 0,2 mM each deoxyribonucleoside triphosphates (dNTP), 10 pmol of each primer, 1 unit Taq polymerase Maxima Hot Start Taq DNA Polymerase (Fermentas) and 150 ng of genomic DNA as template.

PCR was performed in a thermocycler Mastercycler pro S (Eppendorf). The reaction was started by incubating the mixture at 95 ° C for 7 minutes, followed by 30 cycles of incubations: 95 ° C - 30 seconds, 55 ° C for 40 seconds, 72 ° C - 1 min. The final elongation was performed at 72 ° C for 10 minutes.

Amplification products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide. Electrophoresis was performed in a horizontal electrophoresis chamber Bio-RAD Basic, and current source «Consort EV-243". As an electrode buffer used 1hTAE buffer. Documentation of the

results was performed using a gel documentation system Gel Doc. Molecular size of the analyzed DNA samples was determined by comparing their electrophoretic mobility markers - DNA fragment of known molecular weight. As molecular weight markers used O'GeneRuler™ 100bp Plus DNA Ladder production (Fermentas).

PCR products were purified from the residue by oligonucleotide defosfolirirovaniya using alkaline phosphatase (SAP-shimp alkaline phosphatase), and endonuclease I [Werle E., 1994].

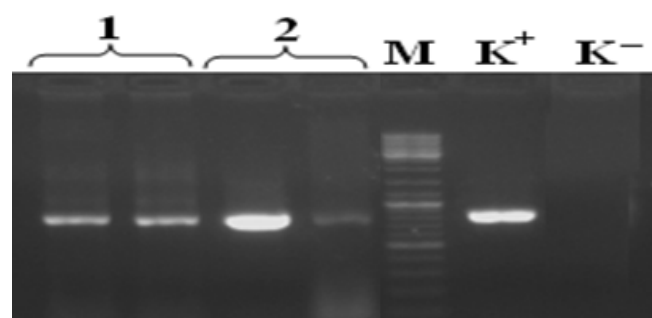
Sequencing of the 16S rRNA gene fragments identified bacteria were performed on an automatic sequencer 3730hl DNA Analyzer (Applied Biosystems, USA) using a set of Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer's recommendations. Results were treated in a program sequencing SeqMan (Applied Biosystems). Search homologous nucleotide sequences of 16S rRNA genes was performed using BLAST (Basic Local Alignment Search Tool) in the Gene Bank database of the National Center for Biotechnology Information US (<http://www.ncbi.nlm.nih.gov>). Identification was carried out with respect to inventory numbers Gene Bank of the first three nucleotide sequences having the best match. Phylogenetic analysis was performed using software MEGA4 [Tamura K., 2007]. Alignment of the nucleotide sequences was performed using the algorithm ClustalW [Thompson J., 1994]. To construct phylogenetic trees using the method of "neighbor-joining" Neighbor-Joining (NJ) [Clarridge J., 2004]. Additionally, the analysis included the nucleotide sequences of closely related species of the phylogenetic group of *Lactobacillus buchneri* and *Lactobacillus plantarum*, deposited in international databases.

RESULTS AND DISCUSSION

Phylogenetic analysis of the nucleotide sequences of the genes 16S rRNA.

In our study of genetic identification of 2 strains was effected by determining the nucleotide sequence of direct 16S rRNA gene fragment, followed by comparison of nucleotide identity with the sequences deposited in the international database Gene Bank, as well as the construction of the phylogenetic tree with reference strains nucleotide sequences.

As a result, isolation of DNA samples were obtained with a high concentration of DNA 80 to 150 ng / ml, the ratio of the wavelengths 260/280 averaged 2.12. By PCR fragment was amplified 16S rRNA gene, a molecular weight of about 800 bp Results of samples with the negative amplification controls are displayed in Figure 1.



Note - 1 strain №22, 2- strain №20, (in duplicate), M molecular weight marker O'GeneRuler™ 100bp Plus DNA (Fermentas) (100 -1000 bp step 100 bp) (K) - negative control PCR (K +) - positive control for PCR

Fig. 1. Elektroforerogramma PCR - products obtained by amplification of the 16S rRNA gene fragment

PCR amplification products were used to determine the nucleotide sequence.

Assessment of nucleotide identity of the analyzed sequences 16S rRNA gene fragment from the reference sequence of phylogenetically closely related species is presented in Table 1.

Table 1. Results Identification of the nucleotide sequence in the international database

Name of the strain	GeneBank accession number	Name of the strain	% matches
Lactobacillus plantarum №22	AB690214.1	Lactobacillus farraginis	100
	KC351904.1	Lactobacillus hilgardii	98
Lactobacillus cellobiosus №20	AB362673	Lactobacillus pentosus.	100
	AJ640078	Lactobacillus plantarum	98

To exclude systematic error analysis of 16S rRNA gene [Rossello-Mora, 2001], was carried out further construction of phylogenetic trees (Fig. 2.3).

After removal of the primer sequences in the non-overlapping areas of the nucleotide sequences were obtained 650-bp. When aligning, it was revealed that the nucleotide sequences of 16S rRNA gene fragment reference strains *Lactobacillus farraginis* (Gene Bank AB690214.1), *Lactobacillus hilgardii* (Gene Bank KC351904.1), *Lactobacillus pentosus* (AV362673), *Lactobacillus plantarum* (AJ640078), and had a size of 560-550 bp respectively. In this regard, for formation of a homogenous sample size of 550 bp was removed from a part of the nucleotide sequences of the test strains.

Percent identity of the nucleotide sequences of fragments 16S rRNA gene between *Lactobacillus plantarum* №22 and *Lactobacillus farraginis* was 100%, and between *Lactobacillus cellobiosus* №20 and *Lactobacillus pentosus* also made - 100%. The nucleotide sequences of 16S rRNA gene identified two cultures *Lactobacillus plantarum* №22, *Lactobacillus cellobiosus* №20 located on the same branch with reference strains *Lactobacillus farraginis* and *Lactobacillus pentosus*.

When identifying a Gene Bank data on crops 16S rRNA highest identity was obtained with reference strains *Lactobacillus farraginis* and *Lactobacillus pentosus*. The cultures by 16S rRNA gene were identified as *Lactobacillus farraginis* and *Lactobacillus pentosus*.

The results of phylogenetic analysis of 16S rRNA gene sequences from strains studied are shown in the phylogenetic tree (Fig. 2-3), which was built in the program MEGA4, using cluster Neighbor-Joining method of calculation of genetic distances and bootstrap analysis, reflecting the accuracy of clustering.

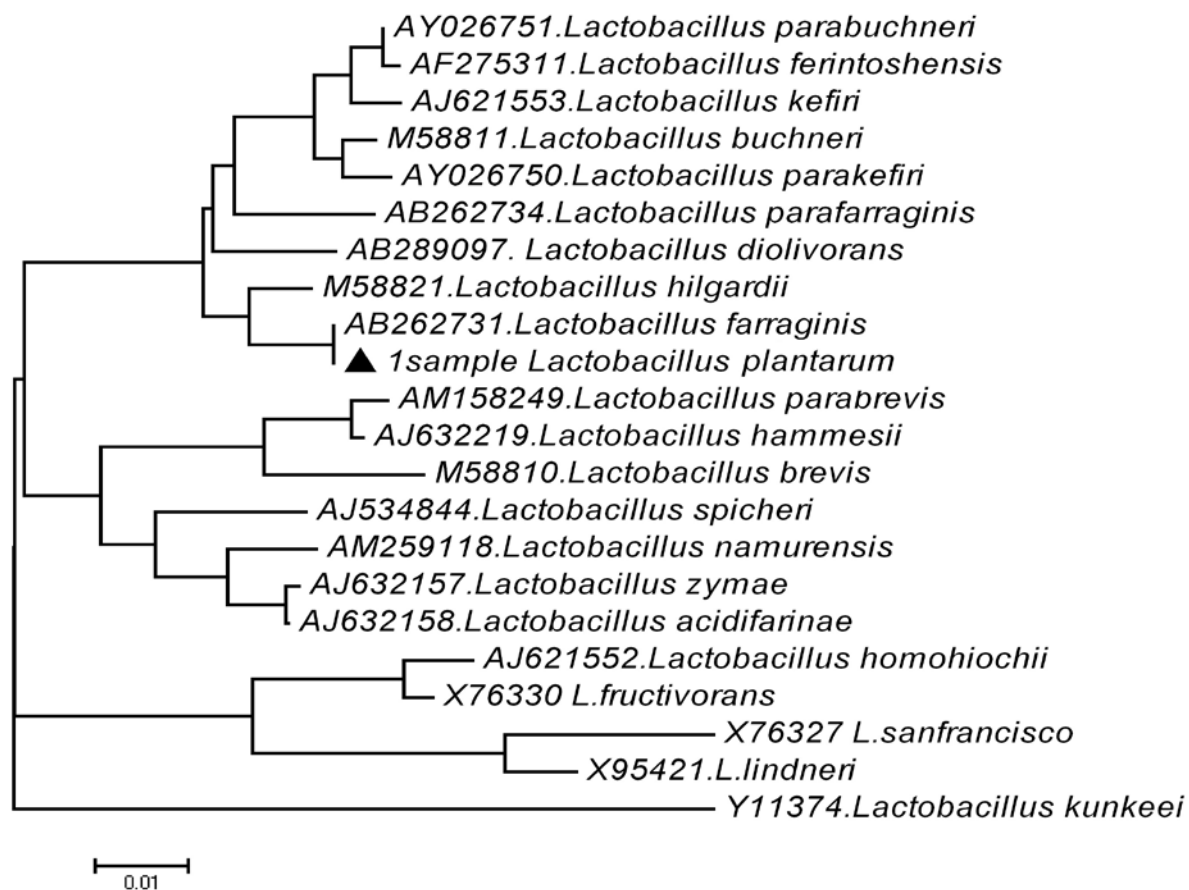


Fig. 2. Phylogenetic tree constructed based on the analysis of the gene fragment 16S rRNA, indicating kinship group *Lactobacillus buchneri*.

As seen in Figure 2, the nucleotide sequence of the strain identified as *Lactobacillus plantarum* № 22, in the identification of a Gene Bank are on the same clade with *Lactobacillus farraginis*. Thus the strain *Lactobacillus plantarum* № 22 on the basis of nucleotide sequence analysis of 16S rRNA gene was identified as *Lactobacillus farraginis*. In general, a cluster of bacteria *Lactobacillus farraginis*, characterized by a high level of variability in gene 16S rRNA, than a cluster formed by *Lactobacillus hilgardii*. The strain *Lactobacillus plantarum* №22 was closely associated with the reference strain *Lactobacillus farraginis*, that allows us to consider it as a phylogenetically closest to the type strains of species *Lactobacillus farraginis*. This is the rationale for the lawfulness of his assignment to *Lactobacillus farraginis* (Figure 3).

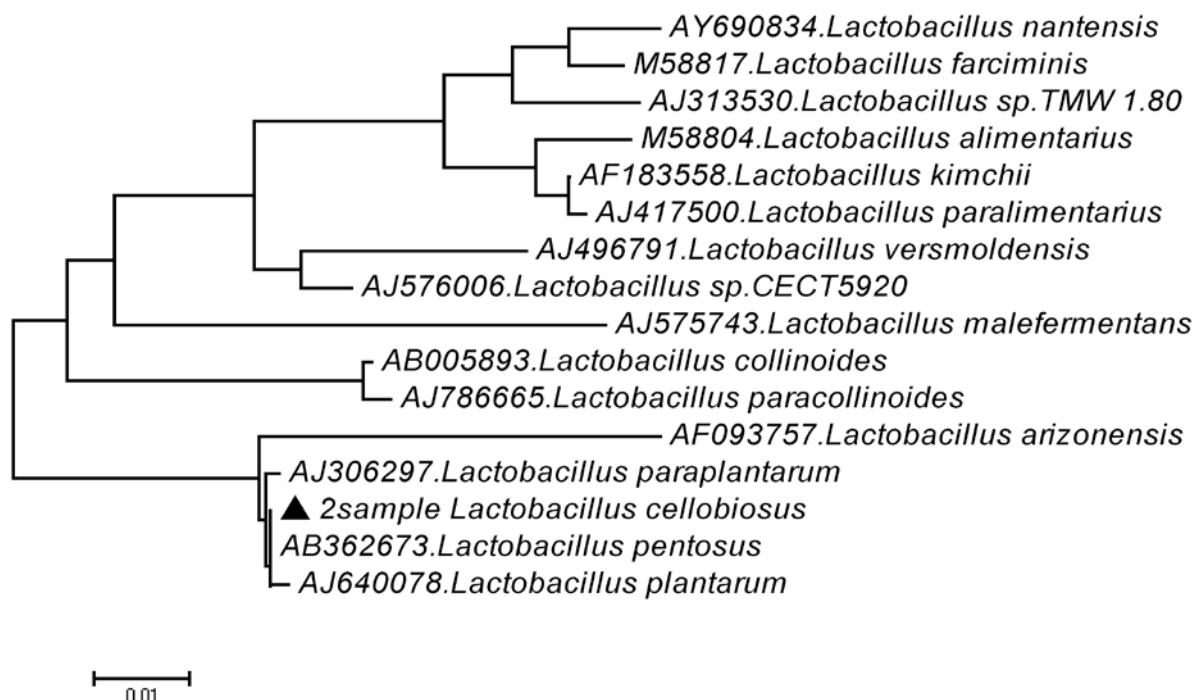


Fig. 3. Phylogenetic tree constructed on the basis of the analysis of 16S rRNA gene fragment shows how the group *Lactobacillus plantarum*.

As seen in Figure 3, phylogenetic analysis fragment 16S rRNA gene strain of *Lactobacillus cellobiosus* № 20 was merged into a single cluster with *Lactobacillus paraplantarum*. When identifying a Gene Bank strain *Lactobacillus cellobiosus* № 20 is located on one clade formed by the bacteria *Lactobacillus pentosus* and *Lactobacillus plantarum*, indicating that it belongs to one of these species, and excluded the possibility of attributing to the *Lactobacillus cellobiosus*. Differentiation of species of *Lactobacillus pentosus* and *Lactobacillus plantarum* based on the results of the phylogenetic analysis was impossible.

The reason for this can be considered a low variability of the nucleotide sequences of the genes 16S rRNA. According to Gene Bank level of genetic relatedness 16S rRNA gene studied culture *Lactobacillus cellobiosus* № 20 in relation to *Lactobacillus pentosus* made - 100%, and is closely related to the most mind *Lactobacillus plantarum* - 98%. In the analysis of phylogenetic tree can be seen that an identifiable culture *Lactobacillus cellobiosus* №20 phylogenetically associated with *Lactobacillus pentosus* and *Lactobacillus plantarum*. Importantly, nucleotide identity between closely related bacteria phylogenetically group *Lactobacillus plantarum*, *Lactobacillus pentosus* and as *Lactobacillus plantarum* was over 98%, which does not allow one to differentiate these types.

Thus, this study shows the results of the molecular genetic identification of two strains of lactic acid bacteria on the basis of nucleotide sequence analysis of 16S rRNA gene, isolated from the gastrointestinal tract of healthy humans. Both strains of lactic acid bacteria were sequenced and analyzed. Comparative analysis of nucleotide sequences of both strains of lactic acid bacteria showed their high identity.

Collectible strains of lactobacilli on the results of our analysis of the sequences of 16S rRNA genes were divided into 2 groups. The first group entered the strain *Lactobacillus buchneri* *Lactobacillus plantarum* №22, species affiliation is not compatible with their original passport data compiled on the basis of traditional microbiological methods. The second group is related strain *Lactobacillus plantarum* *Lactobacillus cellobiosus* №20, whose 16S rRNA gene sequence does not correspond to the

species specified position in the passport data. Comparison of the nucleotide sequences of 16S rRNA genes from collection strains such international databases revealed that strains №22 and №20, previously identified as *Lactobacillus plantarum* №22 strains and *Lactobacillus cellobiosus* №20, actually belong to *Lactobacillus farraginis* and *Lactobacillus pentosus*.

Sequencing of the 16S rRNA gene showed high homology with members of the genus *Lactobacillus*: a strain of *Lactobacillus plantarum* № 22 - 100% with *Lactobacillus farraginis*; a strain of *Lactobacillus cellobiosus* № 20 - 100% with *Lactobacillus pentosus*.

Thus, it is advisable to carry out a genetic analysis on the basis of the identification of the nucleotide sequence 16S rRNA gene, as it allows to identify the species and culture shorten the research.

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