MOLECULAR TAXONOMIC CHARACTERISATION OF PROBIOTIC STRAIN

LACTOBACILLUS SP. 50P1

D. Nikolova¹, Y. Evstatieva¹, R. Georgieva², S. Danova², V. Savov¹, S. Ilieva¹, P. Dalev¹
¹Department of Biotechnology, Biological Faculty, Sofia University St. Kliment Ohridski, Sofia, Bulgaria
²Department of Microbial Genetics, Stephan Angeloff Institute of Microbiology, Bulgarian Acad. Sci., Sofia, Bulgaria
Correspondence to: Dilyana Nikolova
E-mail: dinikolova@mail.bg

ABSTRACT

In recent years interest in the probiotic lactobacilli has been stimulated by the use of these bacteria in products that are claimed to confer health benefits on the consumer. The probiotic effects are usually strain-specific, meaning that a correct identification is important to link the strain to the specific health effect. Taxonomical characterization of probiotic strains only in phenotypic and physiological characteristics is often with low level of discrimination, probably due to their co-evolution in the same ecological niches. Thus, the nucleotide base techniques provide an accurate basis for phylogenetic analysis and identification. With this aim a probiotic strain Lactobacillus spp. 50P1 was studied.

Through the physiological characterization with API 50 CH the strain 50P1, was determined as Lactobacillus helveticus, with low probability- 77.5%. Three PCR-based methods, species-specific PCR, of 16S rRNA gene sequencing, and restriction enzyme of 16S rDNA ARDRA analysis were used for reliable taxonomic characterization of probiotic strain Lactobacillus sp. 50P1. The sequence obtained from the strain was compared to those of Lactobacillus species held in GenBank and the belonging of the strain 50P1 to the species Lactobacillus helveticus was confirmed.

Keywords: Lactobacillus helveticus, Molecular techniques, Taxonomy

Introduction

Lactobacilli are among the most important food bacteria with GRAS statute (Generally Recognized as Safe), with more than 140 species presented. The growing interest on lactic acid bacteria (LAB) application as starters or probiotics in functional products, which are claimed to confer health benefits on consumers, stimulate the scientific research in last years. Thus, the identification and taxonomical characterization of each new selected microorganism at species level is becoming more and more required (13). The large number of species in the genus Lactobacillus, together with their high phenotypic and physiological similarity put there taxonomy often in confusion, and many times leads to misidentifications (6). While, the new molecular identification methods and characterization tools are far more consistent, rapid, reliable and reproducible and can discriminate even between closely related species, which are otherwise indistinguishable on the basis of phenotype (11). According to modern polyphasic taxonomy a combination of various phenotypic and molecular methods have to be used for typing and sub-typing of bacteria. Different molecular techniques, such as Randomly Amplified Polymorphic DNA (RAPD), Ribotyping, PCR - ribotyping, Restriction fragment length polymorphism (RFLP), multiplex PCR and PCR with specific primers, etc., have been applied to detect lactobacilli in various ecosystems (5, 11). Amplified ribosomal DNA restriction analysis (ARDRA) is equally efficient for species level identification among Lactobacillus species of importance (12). In addition, the nucleotide base sequences of Lactobacillus 16S ribosomal DNA (rDNA) provide an accurate basis for phylogenetic analysis and identification. Determination of 16S rRNA gene sequences in variable region V1–V3 has emerged as a viable option for strain identification and phylogenetic analysis (15).

The present work aimed to identify strain Lactobacillus 50P1, by complex of phenotypic and molecular methods for taxonomy characterization. In our recent studies, the cell surface characteristics of strain (9), its activity against pathogenic bacteria (8) and other probiotic properties were characterized. In accordance of the criteria for selection of probiotics, the fully taxonomical characterization of potential probiotic strains is important in health aspect and microbiological safety (10).
**Materials and methods**

**Bacterial strains and growth conditions**

*Lactobacillus sp.* 50P1 from the culture collection of the Department of Biotechnology, Biological Faculty, Sofia University and the reference *Lactobacillus helveticus* ATCC 15009 strains were used in this study. The stock cultures were maintained at -20°C in 20% (v/v) glycerol/MRS broth. Working cultures were prepared from frozen cultures by two successive transfers in MRS broth (Merck KGaA, Germany).

**Phenotypic characterization**

Carbohydrate fermentation profile was obtained by using of commercial API 50 CHL tests according to the manufacturer specification (bioMérieux, France). The apiweb® identification software was used to interpretation of the carbohydrates fermentation results.

**DNA extraction**

Total DNA from *Lactobacillus* strains was isolated as describe by Delley et al. (3).

**Species-specific PCR amplification of 16S-23S rDNA**

All PCR reactions were done with Ready To Go™ PCR beads (Amersham, Biosciences), in 25µl volume, in a Progene cycler (Techne, UK). The 16S-23S intergenic spacer region amplification was carried out according to Tilsalat-Timisjarvi and Alatossava (14), using the primer Hel I - 5’-CTCTTCTCGGTCGCTTG -3’ and Hel II - 5’CTCTTTCTCGGTCGCTTG-3’ (LKB Vetriebs GmbH, Austria) of the 16-23S rRNA of *L. helveticus*. The amplification program used was: 92°C for 2 min, 30 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and 72°C for 2 min. PCR products were visualized in a 1.2% agarose gel by UVP system, after ethidium bromide staining.

**16S rDNA amplification and sequencing**

DNA from the strains *Lactobacillus* sp. 50P1 was amplified using the primer set fD1 and rD1 (Weisburg WG, 1991). Obtained PCR product purified by the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, England) was used as a template DNA for two-time repeated standard sequencing procedure. The analyses were performed in Microgen Ltd, South Korea. The sequencing reactions were carried in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (Applied Biosystems), following the protocols supplied by the manufacturer; (ii) the sequences were determined by ABI PRISM®310 DNA Genetic Analyzer, (PE Applied Biosystems), (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using universal (27F or 1492R) primers. Obtained sequences were edited manually with Chromas version 2•3 (17) and were compared with available sequences in GenBank DNA database using the BLAST algorithm (18).

**16S ARDRA analysis**

The PCR product from 16S rDNA amplification were digested with endonucleases BamH I and HaeIII, following the manufacturer’s instruction (Boehringer Mannheim GmbH, Germany). The restriction patterns were analyzed in 2% agarose gel.

**Results and Discussion**

In present study *Lactobacillus sp*. 50P1 strain isolated from Bulgarian yogurt was identified, according to polyphasic taxonomy. As a first step classical phenotypic characterization was done. The strain was homofermentative, with a temperature optimum at 41°C and high milk coagulation activity. On the base of results from carbohydrate utilization, estimated by API 50 CHL system, *Lactobacillus sp.* 50P1 was classified as species *Lactobacillus helveticus*, with low confidence -77.5%. The API tests are fast and widely used system for physiological characterization and grouping of LAB isolates. The reliability of these tests in the case of philogenetically closely related lactobacilli have been questioned and some controversial results were achieved (2).

Thus, the application of more reliable and discriminative method was necessary and the strain 50P1 was subjected to *L. helveticus* species-specific PCR analysis. The V1–V3 region of 16S rRNA has been found sufficiently variable to provide species-specific patterns in PCR (7). Besides the 16S rRNA gene, the 16S–23S spacer region has also been targeted successfully in PCR for a number of species (1, 13).Obtained species-specific PCR amplification products were identical for the strain 50P1 and for the type strain *L. helveticus* ATCC 15009, used as positive PCR control (Fig. 1A). However, additional 16S ARDRA analyses with HaeIII restriction enzyme revealed a difference in polymorphic ARDRA profiles of both strains (Fig. 1B). Probably, observed difference in HaeIII-ARDRA patterns was due to the intra-species differentiation, reported also for other strains belonging to identical taxonomic group (4).
**Fig. 1.** *L. helveticus* species-specific PCR (A) and comparative 16S ARDRA analyses (B) of *Lactobacillus* 50P1 strain

A) DNA binding patterns obtained by PCR with primers specific for the *L. helveticus*: Lanes: 1- a negative PCR control (no DNA), 2- 100 bp DNA Ladder, 3- *L. helveticus* ATCC 15009 T, 4- *Lactobacillus* sp. 50P1

B) 16S ARDRA restriction patterns of strain *Lactobacillus* sp. 50P1 and reference strain *L. helveticus* ATCC 15009 T.

**Fig. 2.** Phylogenetic tree of *Lactobacillus helveticus* 50P1 created on the base of 16S rDNA sequences A) BLAST in Reference genomic sequences database; B) BLAST in Nucleotide collection database

Determination of 16S rRNA gene nucleotide sequences in variable region V1–V3 has emerged as a viable option for strain identification and phylogenetic analysis. As an alternative, the intergenic spacer region between 16S and 23S rRNA genes containing a variable number of tRNA genes can also be a suitable target for sequencing (11). Application of
golden standard in bacterial taxonomy studies - the 16S rDNA sequencing allowed correct identifying of 50P1, as a strain belonging to the species *L. helveticus* (Fig. 2).

**Conclusions**

The polyphasic taxonomy is a modern approach combining morphological, biochemical and physiological characteristics with molecular based genomic techniques (15). In fact, polyphasic taxonomy has been recognized by the International Committee on Systematic Bacteriology as a reliable approach for description of species and for revision of the present nomenclature of some bacterial groups (11). As well, it allows obtaining correct information on species affiliation and biodiversity of lactobacilli from different habitats. Recently, different *L. helveticus* strains isolated from Bulgarian yogurt were characterized according modern requirement by high discriminative molecular – typing methods (5). Molecular characterization and typing, both genetic and phenotypic, of probiotic strain Lactobacillus sp. 50P1 confirmed it belonging to species *Lactobacillus helveticus*. Using the molecular profile of this strain, it can be suitable for specific technological uses.

**Acknowledgment**

This work was supported by the National Science Fund, Ministry of Education and Science: Project VU-B-7/05.

**REFERENCES**