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SHORT COMMUNICATIONS

Phylogenetic Position and Phosphate Solubilization Activity of Lactic Acid Bacteria Associated with Different Plants

K. M. Zlotnikov[a, b, 1], A. K. Zlotnikov[b], E. N. Kaparullina[a], and N. V. Doronina[a]

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

[b] *Albit Scientific and Industrial LLC, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

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Lactic acid bacteria (LAB) are gram-positive, nonspore-forming (except for some members of the genus *Sporolactobacillus*), catalase-positive bacteria, devoid of cytochromes, aero- and acid-tolerant, which form lactic acid as a final metabolite in the lactic acid fermentation of sugars.

LABs have been found in the nutrient-rich foods (dairy products, meat, and vegetables) and inhabit the digestive system of humans and animals; some LAB species were found on plants [1]. We hypothesize that LABs are imported into human and animal intestines with plants, to which they are probably associated in a form of phytosymbiosis.

In view of the above, the goal of the present work was to isolate pure LAB cultures from different plant species in winter and in summer and to determine their phosphate solubilization activity and phylogenetic position.

Enrichment LAB cultures were obtained as follows: the plant material (leaves and buds) was washed twice with sterile distilled water, ground, and placed into hermetically sealed screw-capped test tubes, filling them to about 80% of their volume. The residual volume was filled with sterile water; the test tubes were hermetically closed and incubated at 30°C in a thermostat for 24 h. The tubes were manually shaken two times during the incubation. After the incubation, the samples from the liquid phase were plated on an agarized nutrient medium 2T5 and incubated in a thermostat at 30°C for 20–40 h.

The 2T5 medium developed in our laboratory contained (per 1 L of H_2O) yeast extract, 5 g; tryptone, 1.5 g; soy peptone, 0.7 g; aminopeptide from animal blood plasma, 20 mL; biomass autolysate (12 g/L by dry substance weight) of the fungus *Cephaliophora tropica* D3, 50 mL; pumpkin juice, 60 mL; tomato juice, 20 mL; pH 6.0.

The agarized (20 g/L agar) 2T5-P medium with calcium phosphate was prepared as follows: equal volumes of hot (50–60°C) agarized 2T5 medium (one with $CaCl_2$ 40 mM, and the other with K_2HPO_4 17 mM) were poured together under continuous agitation and then immediately dispensed into petri dishes. The suspension of water-insoluble calcium phosphates ($Ca_3(PO_4)_2$, $CaHPO_4$, $Ca_5OH(PO_4)_3$) was evenly spread over the volume, making the medium opaque.

Lactic acid in the cultivation medium was detected by HPLC (LKB2150 high-pressure chromatograph (Sweden)) at $\lambda = 210$ nm and a temperature of 24°C on a Reprosil-Pur C8 column (250 × 4.6 mm), with 0.01 N H_2SO_4 as an eluent.

DNA was isolated from 20–30-h cultures grown in 150–200 mL of liquid 2T5 medium at 30°C. DNA was isolated and purified according to Marmur [2]. The 16S rRNA gene was amplified by PCR with the primers 27f: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r: 5'-AAGGAAGGTGATCCAGCTCGT-3' universal for prokaryotic 16S rDNA [3]. The reaction products were separated by electrophoresis in 1% agarose gel. DNA fragments were isolated and purified from low-melting agarose on the columns with Zymoclean Gel DNA Recovery kit (ZymoResearch-Epigenetic, United States) according to the manufacturer's instructions. PCR fragments were sequenced with the BigDye® R Terminator v. 1.1 kit and an ABI PRISM® R genetic analyzer (Applied Biosystems, United States).

Preliminary phylogenetic search of the 16S rRNA gene sequence similarity in the GenBank [NCBI] database was performed using the BLAST software package [http://ncbi.nlm.nih.gov]. For more precise determination of phylogenetic position, the 16S rRNA gene sequences were aligned manually with the nucleotide sequences of taxonomically close reference strains using CLUSTAL W [http://www.genebee.msu.su/clustal] and with the relevant sequences from the latest version of the NCBI Database Project. The rooted phylogenetic tree was constructed by the neighbor-joining method (NEIGHBOR) in the

[1] Corresponding author; e-mail: director@albit.ru

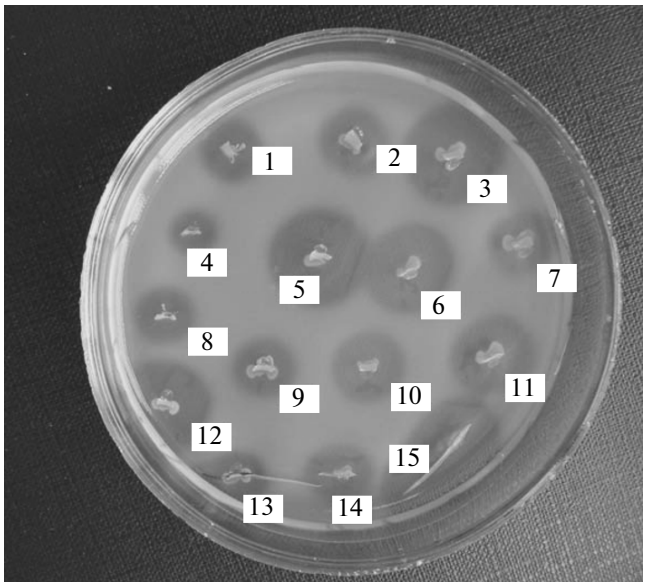

Fig. 1. Phosphate solubilization activity of bacteria on the agarized 2T5 medium with $Ca_3(PO_4)_2$. The isolates of lactic acid bacteria from plants (nos. 1–15), 48 h of growth.

TREECON software package [4]. Evolutional distances were calculated as a number of substitutions per 100 nucleotides. Statistical reliability of branching order was determined by bootstrap analysis of 1000 alternative trees using the respective function of TREECON.

All plant samples were taken in the vicinity of the town of Pushchino, Moscow oblast, in 2012. At the first stage of the work, two LAB strains were isolated from the buds of pine (ALB-C5) and spruce (ALB-E2) in winter (February) after a long period of low temperatures (–25–30°C). Hence, it was supposed that LABs endured severe winter conditions in immobilized state within wood tissues, similar to methylotrophs [5].

At the next stage of the work, LABs were isolated in early May from the leaves of young plants: dill, lettuce, parsley, cabbage, radish, dandelion, goutweed, meadow grass, sorrel, and burdock. It was found out that plating the material from all enrichment cultures on the agarized 2T5 medium resulted in development of mainly slow-growing forms of lactic acid bacteria with colony sizes from barely visible to 0.3 mm, with an admixture of fast-growing microflora (enterobacteria, pseudomonads, bacilli, yeasts, etc.) with the colonies growing up to 1 mm and more in 24 h on the 2T5 medium. The titer of slowly growing bacteria in the liquid phase of enrichment cultures was $0.5–7 \times 10^6$. Under the microscope, they looked like typical cocci or short rods; all of them demonstrated positive Gram stain reaction, did not form spores, grew well in the liquid 2T5 medium without aeration, and accumulated lactic acid, i.e., had the characteristic features of lactic acid bacteria [6].

Judging by the size and morphological characteristics of the LAB colonies on the plates, enrichment cultures were the populations of a few strains (3 to 7 morphologically different colonies could be distinguished). One or several (4 in case of dill and 3 in case of goutweed) typical colonies were chosen from enrichment culture samples of each plant, and the pure LAB cultures were isolated after the repeated standard procedures of microbiological purification (cultivation in the liquid 2T5 medium followed by plating to obtain separate colonies).

The isolates were represented by the following strains: 1, ALB-E2 (isolated from *Picea abiens* (L.) H. Karst. buds); 2, ALB-KR3 (*Urtica dioica* L. leaves); 3, ALB-YK4 (dill *Anethum graveolens* L. leaves); 4, ALB-21 (*Petroselinum vulgare* L. leaves); 5, ALB-YK2 (dill leaves); 6, ALB-YK3 (dill leaves); 7, ALB-YK6 (dill leaves); 8, ALB-C22 (*Brassica oleracea* L. leaves); 9, ALB-C10 (*Rumex acetosella* L. leaves); 10, ALB-1M (*Raphanus sativus* L. leaves); 11, ALB-C5 (*Pinus silvestris* L. buds); 12, ALB-CH1 (glague *Aegopodium podagraria* L.; 13, ALB-LO2 (*Arctium lappa* L. leaves); 14, ALB-109 (*Poa pratensis* L. stems); 15, ALB-91 (*Taraxacum officinale* F.H. Wigg leaves); 16, ALB-CH2 (glague leaves); 17, ALB-CA3 (*Latuca sativa* L. leaves); 18, ALB-CH3 (glague leaves).

All of the isolates were able to dissolve $Ca_3(PO_4)_2$ as was demonstrated by the presence of clearance zones (Fig. 1).

The 16S rRNA gene sequencing showed high levels of homology between some isolates and the members of the genus *Lactobacillus*: the strain ALB-CH2, 99.4% with *L. coryniformis* subsp. *torquens* CECT 4129^T; the strain ALB-CA3, 99.8% with *L. curvatus* DSPV352^T. The strain ALB-CH3 exhibited a 97.7% homology with *L. plantarum* NRRL B-4768^T and *L. pentosus* JCM1558^T and is probably a new species. The strains ALB-CH1, ALB-LO2 and ALB-C5 exhibited 99.5, 99.3, and 98.6% similarity, respectively, with *L. brevis* ATCC 14869^T; the strains ALB-C10 and ALB-C22 had a 99.7% and 99.8% similarity, respectively, with *L. plantarum* NRRL B-14768^T; the strain ALB-91 had a 99.9% similarity with *L. paracasei* subsp. *paracasei* JCM8130^T; and the strain ALB-109 had a 99.4% similarity with *L. zeae* ATCC 15820^T *L. casei* subsp. *casei* ATCC 393^T) (Fig. 2).

Two strains showed high levels of similarity with representatives of the genus *Weissella*: the strain ALB-YK3 had a 98.9% similarity with *W. hellenica* KE3^T and a 98.8% similarity with *W. paramesenteroides* NCFB 2973^T; the strain ALB-YK6 has a 99.8% similarity with *W. viridescens* NRIC 1536^T; and the strain ALB-YK2 had a 99.4% similarity with *Leuconostoc mesenteroides* ATCC 8293^T.

Three strains showed a high level of homology with representatives of the genus *Pediococcus*: the strains

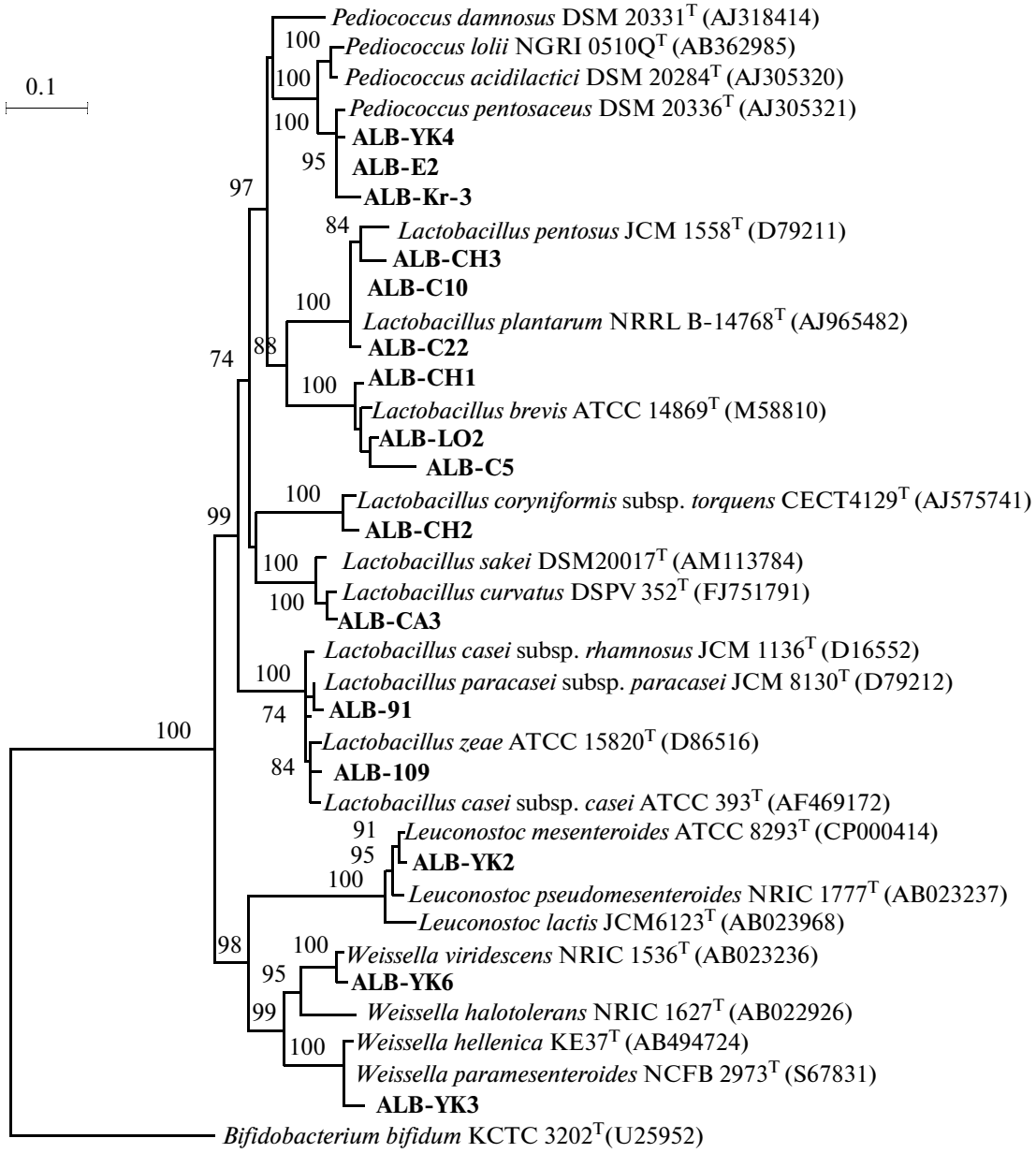

Fig. 2. Phylogenetic positions of the new LAB strains based on the comparison of the 16S rRNA gene sequences. The scale corresponds to 10 nucleotide substitutions per every 100 nucleotides (evolutionary distance). The root was determined by inclusion of the *Bifidobacterium bifidum* KCTC 3202T (U25952) sequence as an outgroup. The numerals show statistical reliability of the branching order determined by bootstrap analysis of 100 alternative trees.

ALB-KR3, ALB-YK4 and ALB-E2 had a 98.9%–99.8% similarity with *P. pentosaceus* DSM 20336T.

Thus, the isolates belonged to four LAB genera: *Pediococcus*, *Leuconostoc*, *Weissella*, and *Lactobacillus*. It should be noted that all four isolates from the dill leaves belonged to different species (*Pediococcus pentosaceus* ALB-YK4, *Leuconostoc mesenteroides* ALB-YK2, *Weissella viridescens* ALB-YK6, and *W. hellenica* ALB-YK3) just as all three isolates from the glague leaves (*Lactobacillus coryniformis* subsp. *torquens* ALB-CH2, *L. brevis* ALB-CH1, and *L. pentosus* ALB-CH3).

The isolation of LAB from different plants during different seasons demonstrates their permanent association with plants. The well-known ability of LAB to synthesize fungicidal compounds [7] and the revealed phosphate solubilization activity suggest their symbiotic connection with plants.

Further studies are necessary to elucidate the mechanisms of interaction between LAB and plants.

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