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RAPD analysis of *Leuconostoc mesenteroides* strains associated with vegetables and food products from Korea

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Lactic acid bacteria play a very important role in food fermentation. For strain identification, characterization and protection, it is important to use highly discriminatory identification methods. In the present study, 37 strains isolated from vegetables and food products of Korea were identified using 16S rDNA gene sequencing and were found to be *Leuconostoc mesenteroides*. Further, molecular characterization of all the strains was performed using two RAPD primers, i.e. 239 and KAY3. All strains have shown RAPD profiles and the amplified products of the profiles ranged from 300 to 4000 bp with both the primers and only small differences in banding pattern were observed. Out of 37, maximum bands were observed in strain 11436 with 239 and strain 11260 with KAY3. With both the primers, the phylogenetic analysis revealed seven clades which could be further subdivided into groups. The dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA). The results showed that 16S rDNA sequencing and RAPD-PCR are suitable preliminary molecular tools for identification and characterization of bacteria.

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1. Introduction

Worldwide, Lactic acid bacteria (LAB) include a large part of potential probiotic microflora. *Leuconostoc*, *Lactobacillus,* and *Pediococcus* are important genera of LAB linked to foods and fermented products such as meat, vegetables, milk products, beverages, bakery products and also act as a flavoring and texturizing agent (O'Sullivan et al., 2002). Genus *Leuconostoc* is a Gram-positive bacteria comprised of 24 different species (De Bruyne et al., 2007). Currently, the type species *Leuconostoc mesenteroides* consists of three subspecies: *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum* and *L. mesenteroides* subsp. *cremoris* (Garvie, 1983). The remarkable fermentative capacity of this genus has been utilized in the enrichment of nutrients, food safety, and has amenable health benefits (Gemechu, 2015). Consumers have accepted that LAB plays an important role in the preservation of food stuff (natural mode) including the health benefits (Quinto et al., 2014). Among LAB, *L. mesenteroides* was first described by Van Tieghem. It is a facultative mesophilic anaerobe, non-motile and non-spore forming in nature and exist in food stuffs, milk products, meat products, wine, and green vegetation. They are obligatory heterofermentative cocci, often ellipsoidal. They usually occur in pairs and chains (Hemme & Foucaud-Scheunemann, 2004). Leuconostocs are used as starter cultures in various industrial processes such as production of buttermilk, cheese, butter, sourdough, kimchi and kefir and also play very important role in food fermentation (Dan et al., 2014). Considering the economic importance, many researchers have used genetic tools to explore LAB at the genetic level. Sequencing analysis of the 16S rDNA gene has been used to determine the diversity of LAB in food (Chen, Yanagida, & Shinohara, 2005; Jung et al., 2011). In the last few years, there is progress in new genomic technologies, which can be utilized for strain selection, use and improvement bestowing industrial needs. Thus, the use of molecular methods is a boon for full identification and characterization of bacterial species (Botina, Tsygankov, & Sukhodolets, 2006). Molecular typing methods include random amplified polymorphic DNA (RAPD-PCR), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), pulse field gel electrophoresis (PFGE), repetitive-element PCR (rep-PCR), single locus sequence typing and multilocus sequence typing (MLST) (Tamang et al., 2005). These molecular techniques are regarded important for specific characterization and detection of LAB strains (Gevers, Huys, & Swings, 2001; Holzapfel, Haberer, Geisen, Björkroth, &

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Schillinger, 2001). The Random amplified polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) is a convenient method for identification and characterization of bacterial strains. Several studies have reported success in using RAPD-PCR method for the differentiation of LAB strains (Ashmaig, Hasan, & Gaali, 2009; Banwo, Sanni, Tan, & Tian, 2012; Cocconcelli, Porro, Galandini, & Senini, 1995; Drake, Small, Spence, & Swanson, 1996; Du Plessis & Dicks, 1995; Nigatu, Ahrne, Gashe, & Molin, 1998; Ruiz, Sesena, & Palop, 2014; Silva et al., 2015; Yang, Z-l., Yang, Huang, & Chen, 2013). The RAPD technique utilizes a short single primer of arbitrary sequence that binds at random (non-specific) locations in the genome (Baker, Crumley, & Eckdahl, 2002). The RAPD profiles generated consisted of repetitive or unique sequences that depends upon homology between template DNA and the primer sequence used (Kumari & Thakur, 2014). The advantages of this technique include general applicability, low cost per reaction, and less technical expertise. In addition, there is no requirement of prior sequence knowledge and for PCR amplification limited quantity of DNA is required. Therefore, by considering the importance of this technique, the objective of the present study was planned to amplify 16S rDNA gene of 37 bacterial strains isolated from different food stuffs of South Korea and finally their characterization using RAPD-PCR technique.

2. Materials and methods

2.1. Bacterial isolates

Thirty-seven bacterial strains were isolated from kimchi (11), radish (10), Chinese cabbage kimchi (4), radish kimchi (2), young radish (4), salted clam (2), salted fish (1), soya bean paste (1), onion (1), and salted small octopus (1). Isolates were recovered from the counting plates in Bromo cresol purple (BCP Plate Count Agar, Eiken Chemical Co., Ltd., Japan). The strains were grown at 37 °C overnight. All cells were harvested by centrifugation and cell pellets were used for DNA isolation (Lee & Lee, 2008).

2.2. DNA extraction

Genomic DNA was isolated using AccuPrep Genomic DNA Extraction kit (Bioneer, Daejeon, Korea) which was performed following the manufacturer's procedure. Bacterial cells were pelleted by centrifugation 3 ml overnight culture at 13,000 rpm for 1 min, the pellet was washed with 1 ml of TE buffer (0.1 M Tris-HCl, 0.01 M EDTA, 1 M NaCl). Five hundred microliters of SET buffer (sucrose, 1 M Tris, 0.5 M EDTA) was added to the pellet and vortexed. Further, 50 μL of a freshly prepared solution of lysozyme (Sigma) and 3 μL of RNase A (Sigma) was added into the pellet and incubated at 37 °C for 1 h in water bath. Twenty-five microliters of SDS (25%, w/v) was incorporated in the solution, gently inverted followed by 25 μL of proteinase K (Bioneer) and mixed by gentle inversion. Incubation was done at 37 °C for 30 min. GC binding buffer of a volume of 400 μL was added to the solution and kept for incubation at 60 °C for 10 min. The resulting solution was treated with 200 μL of isopropanol and homogenized by inversion. The solution was immediately transferred to column tube, centrifuged at 13,000 rpm for 1 min and the supernatant was discarded. The column was washed with 500 μL of washing buffer 1 (Bioneer) which was centrifuged at 13,000 rpm for 1 min and the supernatant was discarded, followed by similar washing with 500 μL of washing buffer 2 (Bioneer) twice. Finally, the column was transferred to 1.5 ml vial and 50 μL of distilled water was added and DNA was eluted by centrifugation at 13,000 rpm for 1 min. The quality of DNA was checked on 0.8% (w/v) agarose gel using 1 × TAE buffer (Sambrook, Fritsch, & Maniatis, 1989).

2.3. 16S rRNA gene sequencing- strain identification

For identification of 37 isolated strains, two universal primer pairs, 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACTT-3′), were used for amplifying the 16S rRNA gene (Park, Cho, Kim, & Lim, 2014). 16S rRNA gene was amplified (94 °C for 2 min; 35 cycles for 94 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 7 min) in a 20 μL reaction volume using Bioneer Premix kit. Amplified products were analyzed using 0.8% agarose gel and purified using the Promega Kit (Gel and PCR cleanup system) following manufacturer's protocol instructions. The amplified DNA was sequenced (Sanger, Nicklen, & Coulson, 1977) using an ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, USA) at the Macrogen sequencing service (Macrogen Inc., Seoul, Korea). The 16S rRNA gene sequence homology search was performed at the National Centre of Biotechnology Information—NCBI, database using BLASTN (http://www.ncbi.nlm.nih.gov/blast).

2.4. Primers for RAPD and PCR conditions

PCR amplifications of 37 Korean *Leuconostoc* isolate were carried out in a thermal cycler (BIORAD). PCR was performed by using two RAPD primers 239 (5′-CTGAAGCGGA-3′) and KAY3 (5′-CTGGCGACTG-3′) at a concentration of 10 μL (Moschetti et al., 1998; Young, Power, Dryden, & Phillips, 1994). The premix kit (Bioneer-10 mM Tris-HCl, pH 9.0, 40 mM KCl, 1.5 mM of $MgCl_{2,}$ 250 μM dNTPs mix, 1 U of *Taq* polymerase) was used for PCR amplification. Two microliters of each primer and 1 μL of template DNA were added into the premix vials and final volume was made 20 μL, by the addition of 17 μL of distilled water. The PCR conditions used for RAPD amplification were as follows-pre-denaturation 94 °C for 4 min, 2 cycles of denaturation at 94 °C for 2 min, annealing at 39 °C for 2 min, extension at 72 °C for 2 min. Thirty-five cycles of denaturation at 95 °C for 15 s, annealing at 39 °C for 15 s, extension at 72 °C for 20 s; final extension step at 72 °C for 7 min. Finally, the amplified DNA was stored at 4 °C.

2.5. Agarose gel electrophoresis and phylogenetic analysis

Amplified products were separated by electrophoresis in 1.2% agarose gel (Seakem, Lonza, USA) containing 0.05 μl mL^{-1} ethidium bromide in 1 × TAE buffer. The gel was visualized and imaged using gel documentation system (BIORAD Gel Doc XR^{+}). The band pattern was analyzed using Quantity One software. Size standardization was confirmed by using 100 base-pair (Takara Bio. Inc., Shiga, Japan) and 1 kb ladders. Phylogenic trees were constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Nei, 1987).

3. Results and discussion

In order to ensure that the study strains belonged to *L. mesenteroides*, 16S ribosomal RNA gene was amplified in all selected 37 strains and sequence was chosen to compare with the reference/type strains of the other species. The BLAST analysis confirmed that all the isolated strains of the present study were *L. mesenteroides*. 16S rRNA gene sequences of *L. mesenteroides* have been deposited in GenBank under accession KX289500 to KX289531. It has been studied that application of 16S or 23S rRNA-targeted oligonucleotide probes are the finest and most dependable approach to identify bacteria on a phylogenetic basis (Amenu, 2014). 16S rDNA has been found to be superior to traditional methods based on phenotypic approaches, which are often unreliable and lack the resolving power to analyze the microbial

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