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Article

Identification of *Lactobacillus* spp. associated with nematodes in peach farm soil

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복숭아 농장 토양에서 Nematodes와 연관된 *Lactobacillus* spp.의 분리 및 동정

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Strains D4 and D5 were isolated from peach-rotten soil during the peach harvest season. The isolates were identified based on morphological and biochemical characterization, and identification was determined by 16S rRNA gene sequencing. Results showed that D4 has high similarity to *Lactobacillus plantarum* ATCC 14917^T and *Lactobacillus pentosus* ATCC 8041^T at 99.05% and 98.98%, respectively. D5 was also similar to *Lactobacillus pentosus* ATCC 8041^T and *Lactobacillus plantarum* ATCC 14917^T at 98.71% and 98.64%, respectively. In contrast, isolates showed differences in carbohydrate utilization in comparison to *Lactobacillus plantarum* ATCC 14917^T and *Lactobacillus pentosus* ATCC 8041^T. In view of this we performed VITEK MS matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis, multiplex PCR fingerprinting, and random amplified polymorphic DNA (RAPD)-PCR to further confirm the identification of D4 and D5. The results of these analyses showed that both strains were most similar to *Lactobacillus plantarum.*

Keywords: *Lactobacillus plantarum*, MALDI-TOF MS, multiplex PCR, peach farm soil, RAPD-PCR

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A group of lactic acid bacteria (LAB) includes the typical genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* and is frequently found in various fermented food and feed such as silage (Herrero *et al*., 1996). As they are reported to be important to maintain human health and to exert probiotic benefits, studies on their probiotic traits have been actively conducted across the world. Traditionally, the organisms have been commonly used as a starter in milk, meats, and vegetable fermentations. They affect the flavor development and preservation of fermented foods. Production of antimicrobial compounds representing bacteriocins from many LAB strains are well documented (Choi *et al*., 2002). In addition, LAB helps reduce diarrhea by the exclusion of causative harmful bacteria in the human gut (Guarner and Malagelada, 2003). LAB are mostly facultative anaerobes, Gram-positive, and metabolize glucose into lactic acid under the absence of oxygen. A number of LAB strains have been recovered from a variety of habitats including animals, plants, the human body, and artificial environments (Miyashita *et al*., 2015).

LAB is particular in its nutrition so that proper carbohydrates, nitrogen compounds, and minerals were supplemented to promote their growth. In soil environments, many bacterial

species tend to release extracellular enzymes including phosphatase, which enables bacteria *in situ* to use organic carbons more easily (Tabatabai and Dick, 2002). Peach fruit is rich in sucrose, glucose, fructose, and sorbitol, and sugars such as maltose, isomaltose, xylose, raffinose, and trehalose particularly in the mature fruit (Cirilli *et al*., 2016). Among the LAB, genus *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, and *Weissella* are frequently found in soil (Chen *et al*., 2005). Many strains of LAB recovered from fresh fruits were reported to be antagonistic to phytopathogenic bacteria, suggesting a possible application as a biocontrol agent (Trias *et al*., 2008). In order to isolate the LAB, soil samples were randomly taken from the peach-rotten soil during the peach-harvesting season in this study.

In a previous study we sampled rotten fruits that had fallen from trees in local Korean farms and we sought to recover LAB and free living soil nematodes (Choi *et al*., 2016). From peach-rotten soil sample we recovered two LAB isolates strains D4 and D5 cohabiting with unidentified soil nematodes. In this current study we identify and characterize these two LAB strains.

Materials and Methods

Isolation and maintenance of lactobacilli

Strains D4 and D5 were isolated from soil samples from a peach farm in Republic of Korea. The collected samples were appropriately diluted serially in sterile saline solution (0.85% NaCl w/v), and isolated using a de Man-Rogosa-Sharpe (MRS; Difco) medium supplemented with 0.005% bromocresol purple (Yakuri), 0.02% sodium azide (Sigma), and 1.5% agar. After spreading, it was cultured for 24 h at 37°C. A single colony was subcultured in MRS medium. For long term storage, it was stored at -70°C in MRS broth containing 35% glycerol (v/v).

Lactic acid bacteria (LAB) strains

All the LAB strains other than the isolates used in this study were purchased from the American Type Culture Collection (ATCC), including *L. plantarum* ATCC 14917^T, *L. pentosus* ATCC 8041^T, *L. acidophilus* ATCC 4356^T, *L. rhamnosus* ATCC 7469^T.

Morphological and biochemical characterization of isolates

Isolated strains were subject to Gram-staining (McClelland, 2001), and examined under 1,000× magnification using an optical microscope (Labophot-2; Nikon). Production of spores following heat-treatment at 80°C for 10 min was examined under a microscope (Oberg *et al*., 2016). Catalase activity was determined by dropping 3% H_2O_2 solution on a fresh-grown colony. Field emission scanning electron microscopy (FE-SEM) was used to examine morphological characteristics. Motility was determined using MRS soft agar (0.4% w/v). Gas production of the isolated strains was determined by using the inverted Durham tube in MRS broth containing glucose. Carbohydrate utilization of the two isolates was determined using the API 50 CH kit (bioMérieux). Strains D4 and D5 cultured at 37°C for 24 h in MRS medium were diluted with API 50 CHL medium and inoculated into each of the API 50 CH strips. Carbohydrate utilization was determined by observing the change in the color of each strip.

Acid and NaCl tolerance

The initial pH of the MRS broth was adjusted to pH 2-10 (at intervals of 1.0 pH) with NaOH and HCl. Growth in NaCl solution (1−10% w/v) was tested for 7 days at 37°C (Bui *et al*., 2011). The test strains were fully activated by transferring three times before use and inoculated 2% (v/v) in 10 ml of MRS broth adjusted pH and NaCl concentrations, respectively. The test was repeated twice.

VITEK MS MALDI−TOF analysis

VITEK MS (bioMérieux) matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was used for the overnight culture spread on the VITEK MS-DS disposable target slide and then treated with 1 μl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution. The pre-treated sample was subjected to the instrumental analysis after air-drying. Identification spectra were automatically produced with MYLA software (bioMérieux). The software has registered above 508 species of bacterial spectra in the database. Percent probability and quantitative value were calculated based on results obtained by comparing the indi-

vidual spectrum with those in the database (Bilecen *et al*., 2015).

Molecular identification and phylogenetic analysis

16S rRNA gene sequencing was performed on strains D4 and D5. Genomic DNA was extracted using AccuPrep Genomic DNA Extraction Kit (Bioneer). The universal primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GG TTACCTTGTTACGACTT-3′) were used (Weisburg *et al*., 1991). 16S rRNA gene sequence was analyzed using BLASTN (Altschul *et al*., 1990), the similarity with the type strain were compared using Eztaxon (Kim *et al*., 2012). Phylogenetic analysis was performed sequence alignment using ClustalW (Thompson *et al*., 1994). Phylogenetic trees were constructed using the neighbor-joining method of MEGA6 program (Tamura *et al*., 2013).

Random amplified polymorphic DNA (RAPD)–PCR assay

Random amplified polymorphic DNA (RAPD)-PCR was performed to distinguish between strains D4 and D5, and closely related strains. PCR was conducted with Applied Biosystems 2720 Thermal Cycler (Waltham). RAPD-PCR was carried out using a primer 1254 (5′-CCGCAGCCAA-3′) (Akopyanz *et al*., 1992). RAPD-PCR was performed in a total volume of 20 μl containing 2 mM $MgCl_2$, 250 μM of deoxynucleotide triphosphate, 1 unit of *Top* DNA polymerase (Bioneer), 50 ng DNA/μl and 20 pM/μl primer 1254 were mixed and performed for total 30 cycles under the following conditions: 5 min at 94°C for initial denaturation, 30 sec at 94°C for denaturation, 1 min at 50°C annealing, 1 min at 72°C for extension and 5 min at 72°C for final extension.

Multiplex PCR assay

Multiplex PCR assay was performed using *rec*A gene-based primers: paraF (5′-GTCACAGGCATTACGAAAAC-3′), pentF (5′-CAGTGGCGCGGTTGATATC-3′), planF (5′-CCGTTT ATGCGGAACACCTA-3′), and pREV (5′-TCGGGATTAC CAAACATCAC-3′). PCR was conducted with Applied Biosystems 2720 Thermal Cycler (Waltham). The PCR mixture contains 1.5 mM $MgCl_2$, 250 μM of deoxynucleotide triphosphate, 1 unit of *Top* DNA polymerase (Bioneer), 5 ng DNA/μl, 10 pM/μl primer paraF, pentF, planF, and pREV, 5 pM/μl planF. Amplification conditions are programed as follows: initial denaturation of 3 min at 94°C for initial denaturation followed by 30 cycles of 30 sec at 94°C for denaturation, 10 sec at 50°C annealing, 30 sec at 72°C for extension and 5 min at 72°C for final extension (Torriani *et al*., 2001b).

Results and Discussion

Morphological and biochemical identification

Strains D4 and D5 were Gram-positive, catalase negative, non-motile, non-sporeforming, rod-shape, and did not produce gas by using glucose as a carbon source. Using field emission scanning electron microscopy (FE-SEM) for morphological characteristics, D4 and D5 were rod-shaped with a dimension of 0.5–0.6 × 1.0–1.2 μm (Fig. 1A), 0.5–0.6 × 1.2–1.4 μm (Fig. 1B),

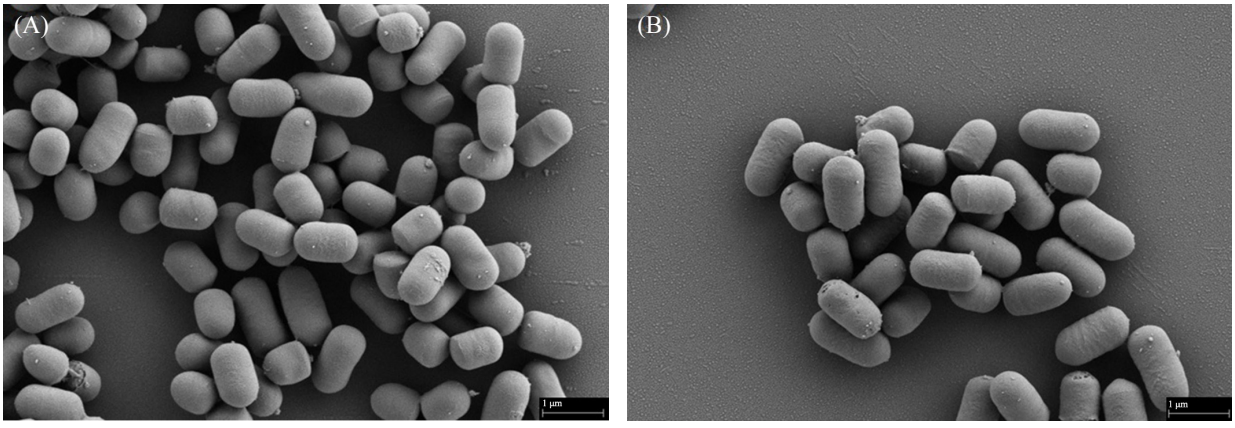

Fig. 1. Scanning electron micrographs of strains. (A) D4 and (B) D5. Bars, 1 μm.

respectively. The isolates were grown in MRS medium adjusted to the initial pH 4–9 and NaCl 1–9% (w/v). Carbohydrate utilization tests with an API 50 CH kit, showed that strain D4 produced acid using L-arabinose, ribose, D-xylose, glucose, fructose, N-acetyl-glucosamine, esculin, cellobiose, maltose, melibiose, sucrose, trehalose, raffinose, gentiobiose, D-turanose, and 5-ketogluconate as a carbon source. Strain D5 was able to grow on several carbon sources: ribose, galactose, glucose, fructose, mannose, mannitol, α-methyl-D-mannoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, sucrose, trehalose, gentiobiose, D-turanose, and 5-ketogluconate (Table 1). Thus strain D4 differed from D5 in that it was able to use xylose, arabinose, melibiose, and raffinose whereas strain D5 was able to use galactose, mannose, mannitol, α-methyl-D-mannoside, amygdalin, arbutin, and salicin. In addition, strains D4 and D5 showed differences compared to *L. plantarum* ATCC 14917^T and *L. pentosus* ATCC 8041^T in carbohydrate utilization using the API 50 CH kit. In contrast to a typical *L. plantarum* subsp. *plantarum* ATCC 14917^T and a *L. pentosus* ATCC 8041^T, both of which grew on lactose, sorbitol, and melezitose, D4 and D5 were unable to use the three kinds of sugars. Among LAB species, *L. plantarum*, *L. pentosus*, and *L. paraplantarum* were very close in terms of the acid producing patterns from similar carbon sources. *L. pentosus* was differentiated from *L. plantarum* in fermenting capability of glycerol and xylose in that few *L. plantarum* strains can use glycerol as a fermentable sugar (Zanoni *et al*., 1987). Those two species were technically difficult to differentiate based on morphological characteristics. *L. plantarum* was also observed to have different substrate utilization and stress response depending on the isolation source (Siezen *et al*., 2010).

Table 1. Differential traits of strains D4 and D5 and strains of closely related species

Items	Lactic acid bacteria*					
	1	2	3	4	5	6
Catalase	-	-	-	-	-	-
Growth with NaCl (%) at:						
9	+	+	D	+	D	+
10	-	W	W	+	W	+
Growth pH at:						
4	D	D	D	D	D	D
10	-	-	-	-	-	-
Acid production from:						
Glycerol	-	-	-	D	W	D
L-Arabinose	+	-	+	-	+	-
D-Xylose	+	-	-	+	-	-
Galactose	-	+	+	+	+	+
Mannose	-	+	+	+	+	+
Mannitol	-	+	+	+	+	+
Sorbitol	-	-	+	+	+	+
α-Methyl-D-Mannoside	-	+	+	+	+	-
Amygdalin	-	+	+	+	+	+
Arbutin	-	+	+	+	+	+
Salicin	-	+	+	+	+	+
Lactose	-	-	+	+	+	+
Melibiose	+	-	+	+	+	-
Melezitose	-	-	+	+	+	+
Raffinose	+	-	+	+	+	-
Gluconate	-	-	+	+	+	+

+, positive; -, negative; W, weakly positive (weak reaction recorded after 7 days); D, delayed (reaction recorded after 3 days).
*1, strain D4; 2, strain D5; 3, *L. plantarum* ATCC 14917^T; 4, *L. pentosus* ATCC 8041^T; 5, *L. acidophilus* ATCC 4356^T; 6, *L. rhamnosus* ATCC 7469^T.

Based on growth characterizations and morphology we initially speculated that D4 and D5 were divergent from the LAB strains *L. plantarum*, *L. pentosus*, *L. acidophilus*, and *L. rhamnosus*. To verify this, we used VITEK MS MALDI-TOF MS analysis. MALDI-TOF MS is a method that can be used to identify bacteria and fungi quickly and accurately by analyzing protein spectra (Foster, 2013). Somewhat surprisingly, VITEK MS MALDI-TOF analysis indicated that strain D4 was identified as *L. plantarum* or *L. paraplantarum* and strain D5 as *L. paraplantarum*.

Molecular characterization and phylogenetic analysis

To verify the MALDI-TOF MS analysis we molecularly characterized D4 and D5. 16S rRNA gene sequences of isolates D4 and D5 were uploaded in Eztaxon (http://eztaxon-e.ezbiocloud.net/) for phylogenetic analysis. The GenBank (http://www.ncbi.nlm.nih.gov/genbank/) accession numbers for the partial 16S rRNA gene sequences of the former and the latter are KU851254, KU851255, respectively. Both of them have above 98% similarity to the type strains of *L. plantarum* ATCC 14917^T, *L. pentosus* JCM 1558^T (= ATCC 8041^T), *L. paraplantarum* DSM 10667^T, and *L. plantarum* subsp. *argentoratensis* DKO 22^T. *L. plantarum* and *L. pentosus* are apparently similar organisms based on their 99% over similarities

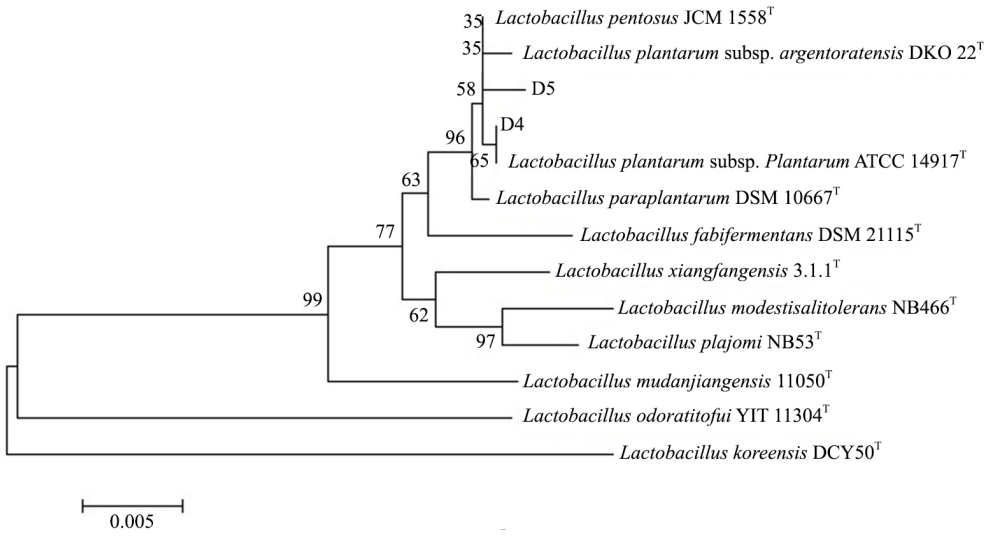

Fig. 2. Neighbour-joining trees based on 16S rRNA gene sequences showing the phylogenetic relationships among strains D4 and D5, and related type strains. Bar, 0.005 substitutions per nucleotide position. Neighbour-joining phylogenetic tree was generated with bootstrap trials of 1,000.

in the 16S rRNA gene sequence (Collins *et al*., 1991). A phylogenetic tree for the D4 and D5 was constructed based on the 16S rRNA gene sequences in the database (Fig. 2). When compared in similarity, D4 is close to *L. plantarum* ATCC 14917^T, *L. pentosus* JCM 1558^T (= ATCC 8041^T), *L. plantarum* subsp. *argentoratensis* DKO 22^T, *L. paraplantarum* DSM 10667^T, and D5 to *L. pentosus* JCM 1558^T (= ATCC 8041^T), *L. plantarum* ATCC 14917^T, *L. plantarum* subsp. *argentoratensis* DKO 22^T, and *L. paraplantarum* DSM 10667^T in decreasing order.

Genomic fingerprinting

RAPD-PCR is commonly used to differentiate *L. plantarum* and *L. pentosus* and other closely related bacterial species (Van Reenen and Dicks, 1996). The results obtained by using a primer 1254 for RAPD-PCR showed that D4 and D5 were very similar to *L. plantarum* ATCC 14917^T but different from *L. pentosus* ATCC 8041^T, *L. acidophilus* ATCC 4356^T, and *L. rhamnosus* ATCC 7469^T in their fingerprinting patterns. It should be noted that a distinct band between 200–300 bp was apparent for isolate D5, strongly indicating that it is different from *L. plantarum* ATCC 14917^T (Fig. 3). Previous study reported that the RAPD-PCR fingerprinting patterns of *L. plantarum*, *L. pentosus*, and *L. paraplantarum* were distinct among the LAB species tested, claiming that species-specific patterns do exist (Torriani *et al*., 2001a). To further confirm the

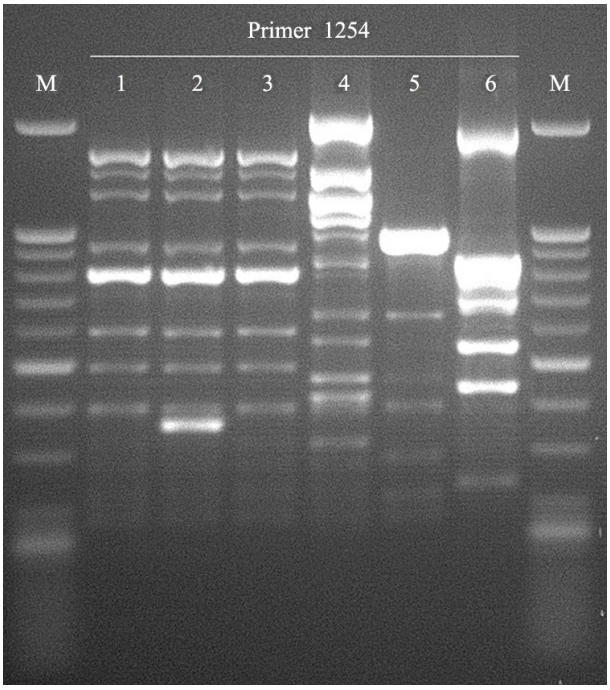

Fig. 3. RAPD-PCR fingerprints of strains D4 and D5, and closely related members of the genus *Lactobacillus*. Lanes: 1, strain D4; 2, strain D5; 3, *L. plantarum* ATCC 14917^T; 4, *L. pentosus* ATCC 8041^T; 5, *L. acidophilus* ATCC 4356^T; 6, *L. rhamnosus* ATCC 7469^T; M, size marker (25/100 bp DNA ladder; Bioneer).

identification of D4 and D5 multiplex PCR assay was performed using individual *rec*A gene-based primer: paraF specific for *L. paraplantarum*, pentF for *L. pentosus*, and planF for *L. plantarum*. The amplicon size for *L. plantarum*

corresponds to 318 bp, *L. pentosus* to 218 bp, and *L. paraplantarum* to 107 bp (Torriani *et al*., 2001b). For strains D4 and D5, there was a 300 bp of amplicon from the multiplex PCR assay, compared to 200 bp amplicon for *L. pentosus* ATCC 8041^T, and none for *L. acidophilus* ATCC 4356^T and *L. rhamnosus* ATCC 7469^T as the negative controls (Fig. 4).

In conclusion, we isolated two strains of *Lactobacillus* spp. from peach-rotten soil and conducted morphological and biochemical characterizations and molecular identification. Taken together, we designated D4 and D5 as strains of *L. plantarum*. Many *Lactobacillus* species are found only in limited habitats. In most cases, *L. acidophilus*, *L. reuteri*, and *L. rhamnosus* are found in the gastrointestinal (GI) tract (Siezen and Wilson, 2010). In contrast, *L. plantrum* is found in a variety of habitats such as vegetables, fish, meat, dairy products and the GI tract (Siezen and van Hylckama Vlieg, 2011). Tanganurat *et al*. (2009) reported that 10 different *L. plantarum* strains could ferment galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine and treharose. Strain D4, on the other hand, does not use galactose, mannose, and mannitol. Furthermore, *L. plantarum* isolated from the same ecological niches have similar genetic diversity and population structure (Xu *et al*., 2015). The natural ecological niche that D4 and D5 were isolated from differs from other *L. plantarum* niches in that multicellular predators such as nematodes are abundant in rotting fruit. In a previous study from which we first isolated D4 and D5, we showed that *C. elegans* can smell wild *L. plantarum* when grown on citrus fruit due to its production of diacetyl (Choi *et al*., 2016). Thus strains D4 and D5 which we found in rotting fruit may also be sensed and consumed by nematodes. Predator-prey relationships like this can result in evolutionary arms races that result in diverse changes at the molecular level and may be a major factor in why such diversity exists in *L. plantarum.*

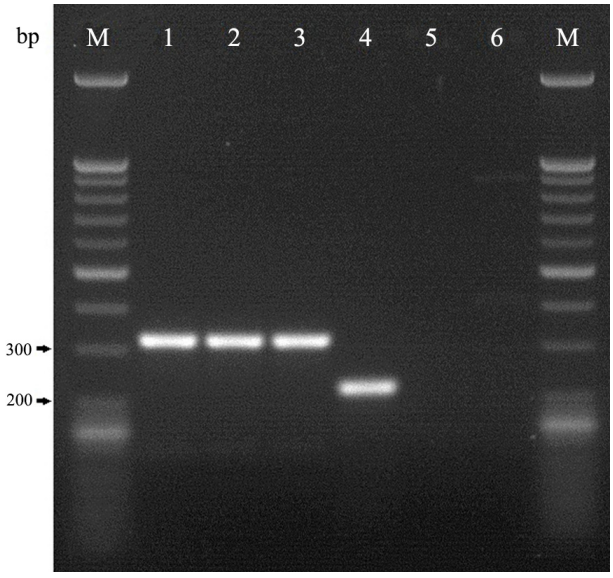

Fig. 4. *Rec*A multiplex PCR fingerprints of strains D4 and D5, and closely related members of the genus *Lactobacillus*. Lanes: 1, strain D4; 2, strain D5; 3, *L. plantarum* ATCC 14917^T; 4, *L. pentosus* ATCC 8041^T; 5, *L. acidophilus* ATCC 4356^T; 6, *L. rhamnosus* ATCC 7469^T; M, size marker (25/100 bp DNA ladder; Bioneer).

적 요

복숭아 수확시기에 낙과한 토양에서 *Lactobacillus* sp. D4와 D5 균주를 분리하였다. 분리한 *Lactobacillus* sp. D4와 D5 균주를 동정하기 위하여 형태학적 동정, 생화학적 동정 및 16S rRNA 유전자서열 분석을 수행하였다. 16S rRNA 유전자서열 분석 결과 *Lactobacillus* sp. D4는 *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^{T}과 *Lactobacillus pentosus* ATCC 40997^{T}에 각각 99.05%, 98.98% 일치하였으며, *Lactobacillus* sp. D5는 *Lactobacillus pentosus* ATCC 40997^T, *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^{T}에 각각 98.71%, 98.64% 일치하였다. *Lactobacillus* sp. D4와 D5 균주는 당 이용성 비교에서 *Lactobacillus plantarum* ATCC 14917^{T}과 *Lactobacillus pentosus* ATCC 8041^{T}에 비교하여 다른 결과를 나타내었다. 정확한 동정을 위하여 VITEK MS matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) 분석, multiplex PCR, random amplified polymorphic DNA (RAPD)-PCR을 수행하였다. 이러한 결과에 근거하여 *Lactobacillus* sp. D4와 D5 균주는 *Lactobacillus plantarum*으로 동정되었다.

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