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Development of species-specific PCR primers and polyphasic characterization of *Lactobacillus sanfranciscensis* isolated from Korean sourdough

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ABSTRACT

Lactobacillus sanfranciscensis is a bacterium used in sourdough that provides desirable properties such as better flavor and texture to the sourdough bread. Here, the intra-species diversity of *L. sanfranciscensis* strains isolated from Korean sourdough was studied using genotypic (multiplex-RAPD-PCR: multiplex-Randomly Amplified Polymorphic DNA-polymerase chain reaction) and phenotypic (VITEK2 Compact system) analyses. For this, a novel species-specific set of PCR primers was developed to identify *L. sanfranciscensis* using the recently published genome database. The primers were able to detect *L. sanfranciscensis* isolated from Korean sourdough with 100% accuracy. Genotyping and phenotyping analyses at the strain level demonstrated that Korean sourdough possesses various biotypes of *L. sanfranciscensis* strains. These strains were clustered into 5 subtypes (genotyping) or 7 subtypes (phenotyping). In summary, this strategy to construct novel primers reduced the chance of cross amplification and was able to identify the desired strain. The various strains isolated in this study can be used to develop a sourdough starter after the analysis of their fermentation characteristics.

1. Introduction

Sourdough is a flour (made from grains such as wheat, rye, or rice) and water mixture fermented by lactic acid-producing bacteria (LAB) and yeasts (De Vuyst and Vancanneyt, 2007; Ravyts and De Vuyst, 2011; Vogel et al., 1999). Fermenting sourdough positively influences the dough's properties by improving its volume, texture, and flavor (Corsetti et al., 1998; Hansen et al., 2004; Vrancken et al., 2011). The nutritional value of the bread also increases because it slowly goes stale and is protected from mold and bacteria that can cause spoilage (Chavan and Chavan, 2011). In sourdough fermentation, LAB are regarded as the key microorganisms. Most of the properties of sourdough result from LAB metabolism: lactic acid fermentation, proteolysis, synthesis of volatile compounds, and production of anti-microbial agents (Choi et al., 2012; Leroy and De Vuyst, 2004). While there are many sourdough LAB, *Lactobacillus sanfranciscensis* in particular has an outstanding effect on the flavor and physical aspects of the bread (Gobbetti and Corsetti, 1997). This strain was first discovered by Kline and Sugihara (1971) and isolated from San Francisco sourdoughs. Studies characterizing its coexistence with *Saccharomyces exiguus* provided a better understanding of its competitiveness in a sourdough environment (Kline and Sugihara, 1971).

As molecular biology techniques have been developed, several groups have worked to isolate *L. sanfranciscensis* for extensive studies. Among the new techniques, fast and convenient methods for detecting 16S rRNA non-conserved gene sequences (Zapparoli and Torriani, 1997) or the 16S–23S intergenic spacer regions (Valcheva et al., 2007) have been developed using specific PCR primers. However, these primers can be sensitive to subtle changes in annealing temperature and often resulted in cross amplification, which prevents accurate species identification. To identify a particular species of interest, it would be more precise to target a species-specific gene for amplification. However, in the past, specific gene targeting for *L. sanfranciscensis* was limited owing to the lack of information about its genome sequence. Recently, the whole genome sequence of *L. sanfranciscensis* TMW 1.1304 was published (Vogel et al., 2011). This allowed us to find species-specific sequences to use for designing a primer set for fingerprinting analysis.

Prior to evaluating novel and naturally occurring microorganisms for use as sourdough fermentation starters, strain-level differentiation strategies should be used for categorizing candidate microorganisms to reduce the labor cost. To accomplish this, a combination of genotypic and phenotypic assays is strongly recommended. There are several

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different genotyping tools that have been used for screening, including Randomly Amplified Polymorphic DNA (RAPD) (Corsetti et al., 2003; Venturi et al., 2012; Zapparoli et al., 1998), pulsed field gel electrophoresis (PFGE) (Zapparoli et al., 1998), amplified ribosomal DNA restriction analysis (ARDRA) (Foschino et al., 2001), and rep-PCR (PCR amplification of repetitive bacterial DNA elements) analysis (De Angelis et al., 2007). In addition to genotyping, phenotypic studies should also be conducted to consider carbohydrate metabolism, proteinase activity, and peptidase activity of candidate microorganisms (De Angelis et al., 2007).

In this study, a rapid and accurate species-specific PCR assay was developed for *L. sanfranciscensis* by using the genome database for *L. sanfranciscensis* TMW 1.1304 (Vogel et al., 2011). The accuracy of this PCR method was evaluated by identifying type cultures of *L. sanfranciscensis*. The species-specific fingerprinting method was used to identify various strains isolated from Korean sourdough. The isolated strains were then differentiated at the strain level to study the intra-species diversity of *L. sanfranciscensis* using genotyping (multiplex-RAPD PCR) and phenotyping (VITEK2 Compact) techniques. Since a long time ago in Korea, a traditional sourdough has been used to make a sponge-like cake (Cho et al., 1994). To prepare the sourdough, nuruk, a wheat flour preparation that is inoculated with environmental microorganisms such as fungi, yeasts, and LAB, is mixed with rice (or wheat) flour and incubated (Kim et al., 2010). To date, there have been no experimental studies carried out to isolate and characterize the strains of *L. sanfranciscensis* from Korean sourdough.

2. Materials and methods

2.1. Microorganisms, growth and storage conditions

Table 1 lists the microorganisms and primers used in this study. The *L. sanfranciscensis* strains were grown at 30 °C in SDB broth (2% maltose, 0.3% yeast extract, 1.5% fresh yeast extractives, 0.03% Tween 80, and 0.6% trypticase at a final pH of 5.6) as described by Kline and Sugihara (1971). The other bacterial strains were grown at 37 °C in MRS (de Man Rogosa and Sharpe, Difco). For isolation of bacterial strains from sourdough, SDB agar (1.5–2% Bacto™ Agar, Difco) plates were used. All the microorganisms were stored at −80 °C in the same medium supplemented with glycerol (15% v/v) until further use.

Table 1
Strains and primers used in this study.

Species/primer	Strain designation/sequence	Source
Strains		
Lactobacillus sanfranciscensis	ATCC 27651	San Francisco sourdough
Lactobacillus sanfranciscensis	JHS 55	This study
Lactobacillus sanfranciscensis	JHS 101	This study
Lactobacillus plantarum	ATCC 8014	N.I.
Lactobacillus brevis	DSM 6235	Spoiled beer
Lactobacillus paracasei	ATCC 25302	Milk products
Lactobacillus sakei subsp. *sakei*	ATCC 31063	Pickled cabbage
Lactobacillus casei	ATCC 393	Cheese
Lactobacillus lactis subsp. *lactis*	ATCC 19435	N.I.
Pediococcus pentosaceus	ATCC 33314	Sake mash
Leuconostoc mesenteroides subsp. *mesenteroides*	ATCC 9135	N.I.
Lactobacillus crustorum	KACC 16344	Artisan wheat sourdough
Leuconostoc citreum	ATCC 49370	Honeydew of rye ear
Lactobacillus curvatus	ATCC 25601	Milk
Lactobacillus paralimentarius	JCM 10415	Japanese sourdough
Lactobacillus buchneri	ATCC 4005	Tomato pulp
Primers		
Sanhyp1	GGAGGAAAACTCAT GAGTGTTAAG	This study
Sanhyp2	CAAAGTCAAGAAGT TATCCATAAACAC	This study
OPL-05	ACGCAGGCA	Seseña et al. (2005) and Venturi et al. (2012)
RD1	GCTTAAGGAGGTGA TCCAGCC	Venturi et al. (2012) and Weisburg et al. (1991)
P1	ACGCGCCCT	De Angelis et al. (2001) and Venturi et al. (2012)

ATCC — American Type Culture Collection, Rockville, Maryland, USA.
DSM — Deutsche SammlungfürMikroorganismen und Zellkulturen. Braunschweig, Germany.
KACC — Korean Agricultural Culture Collection, Suwon, Korea.
JCM — Japan Collection of Microorganisms, Tsukuba, Japan.
N.I. — not informed.

2.2. Genomic DNA preparation

Microorganisms for extracting the genomic DNA (gDNA) were grown using the appropriate medium and temperature under anaerobic conditions until the cell number reached 10^8–10^9. Cells were centrifuged at 13,000 rpm for 1 min and the supernatants were discarded. The chromosomal DNA was extracted using a gDNA prep kit (SolGent, Korea) and the DNA purity was measured by Nanovue plus (GE Healthcare Life Science, USA).

2.3. Specific PCR detection of L. sanfranciscensis

The gene LSA_02510 encoding a hypothetical protein was chosen to specifically identify *L. sanfranciscensis.* A primer set, Sanhyp1 and Sanhyp2, corresponds to positions 37–60 bp and 967–993 bp respectively of LSA_02510 as listed in Table 1. The bacterial strains in Table 1 were used for species-specific PCR reactions under the following conditions: pre-denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min.

2.4. Isolation and identification of L. sanfranciscensis

Korean sourdough was prepared by mixing wheat flour with nuruk, which contained various microorganisms after being spontaneously inoculated from the environment. After incubation, 10 g of the sourdough sample was diluted using a 0.85% NaCl solution and homogenized by a stomacher (AES, France). The mixed samples were serially diluted in the 0.85% NaCl solution and plated on SDB agar. Colonies were picked randomly and gDNA was prepared for species-specific PCR and molecular studies. The gDNA samples isolated from 53 resulting bacterial strains were used to evaluate the accuracy of the *L. sanfranciscensis*-specific PCR assay. The identity of the presumptive *L. sanfranciscensis* and non-*L. sanfranciscensis* strains ascertained using the species-specific PCR assay was confirmed using 16S rRNA gene sequencing (with 27F and 1492R universal primers) (Solgent, Korea).

2.5. Multiplex-RAPD (Randomly Amplified Polymorphic DNA) PCR

To differentiate *L. sanfranciscensis* at a strain level, multiplex-RAPD-PCR designed by Venturi et al. (2012) was conducted with the prepared gDNAs. Primers for OPL-05 + RD1 and P1 + RD1 combinations were used in the multiplex-RAPD-PCR reaction (Table 1). The PCR reaction was performed in a final volume of 20 μL using the Accupower™ PCR premix (Bioneer Co., Korea), and 10 pmol of each primer and the template gDNA in the GeneAmp PCR system 2400 (Applied Biosystems, USA). After the initial pre-denaturation step at 94 °C for 5 min, 40 cycles of PCR amplification were performed using the following conditions: 94 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. To analyze the resulting PCR products, a 2% (w/v) agarose gel was run in a TEB buffer at 100 V for 90 min and

the gel images were captured in an i-MAX™ Gel Image Analysis System (CoreBio, Korea). BioNumerics version 7.1 software (Applied Math, Belgium) was used to convert and analyze the band patterns. Two RAPD profiles were combined and compared using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and Dice similarity coefficient methods (Zapparoli et al., 1998). Multiplex-RAPD-PCR reactions were run in duplicate to confirm the results.

2.6. VITEK2 Compact analysis

VITEK2 Compact (bioMerieux, France) is an automated system able to identify microorganisms by testing 43 biochemical properties in 8 h or less. It can also handle up to 30 samples in one reaction, thus, reducing the time and effort required. VITEK2 Compact was used for this study to differentiate 45 *L. sanfranciscensis* isolates at a strain level by analyzing and comparing the phenotypes. Strains were individually grown on SDB agar. Colonies were picked and mixed in a 0.45% NaCl solution until the McFarland standard measured 0.50–0.63 on the VITEK 2 DensiCheck instrument (bioMerieux). Gram-positive (GP) Colorimetric Identification Cards (bioMerieux) and the tubes containing the bacteria were assembled in a cassette and assayed using the VITEK2 Compact system. Data were analyzed using the VITEK 2 software version VT2-R03.1. Clustering analyses based on phenotype were performed using the method described above (Multiplex-RAPD-PCR).

2.7. Calculation of discriminatory power and biodiversity

Simpson's Index of Diversity (D) was used to calculate the discriminatory power of both genotypic and phenotypic analysis methods (Hunter and Gaston, 1988). The biodiversity of the *L. sanfranciscensis* strains in Korean sourdough was estimated based on the Diversity Shannon Index (H) (Shannon and Weaver, 1949).

3. Results and discussion

3.1. Development of L. sanfranciscensis-specific PCR

To design an *L. sanfranciscensis*-specific PCR primer set, a gene uniquely existing in *L. sanfranciscensis* was selected from the *L. sanfranciscensis* TMW 1.1304 genome database (KEGG, The Kyoto Encyclopedia of Genes and Genomes). The BLASTn (nucleotide BLAST) program (available at http://blast.ncbi.nlm.nih.gov) was used to screen candidate genes based on sequence similarity. The selection criteria were optimized for both highly similar sequences (megablast) and more dissimilar sequences (discontinuous megablast). Several candidate genes were used to match in the megablast search. The hypothetical protein gene LSA_02510 was selected as the target gene for detecting *L. sanfranciscensis* because it had no sequence match with any other bacteria. The discontinuous megablast searches with the LSA_02510 gene did identify several similar sequences in other species (Fig. 1). However, the total scores from the searches with the query sequence were relatively low (*L. sanfranciscensis* = 1824; other species ≤ 230).

Sanhyp1 and Sanhyp2 primers were designed based on the alignment of LSA_02510 with similar sequences. Briefly, both ends of the LSA_02510 gene, indicated with boxes (Fig. 1), were chosen for PCR amplification because the primer set was expected to amplify only LSA_02510 from *L. sanfranciscensis*. The verification of the species-specific PCR assay was performed using various strains of *L. sanfranciscensis* and other type cultures of LAB (Table 1). The results of the species-specific PCR are shown in Fig. 2. In lanes 1–3, the expected 957-bp band was amplified from the *L. sanfranciscensis* strains but no bands were detected from other reference species (lanes 4–16). These results indicated that the newly designed primer set could be used to selectively detect *L. sanfranciscensis*.

Species-specific PCR primers for *L. sanfranciscensis* had been previously designed against the non-conserved regions of the 16S rRNA gene (Zapparoli and Torriani, 1997) or 16S–23S intergenic spacer regions (Valcheva et al., 2007). In contrast, in this study, we chose to use a unique gene identified from the *L. sanfranciscensis* genome database. This enabled us to exclude any possibility that genes from other species would mistakenly be amplified.

3.2. Isolation of L. sanfranciscensis from Korean sourdough

In total, 53 colonies grown on SDB agar plates were used to evaluate the accuracy and applicability of the newly developed PCR primer set and to isolate *L. sanfranciscensis* for further study. Of the 53 colonies, 34 had detectable bands (957 bp) (data not shown) that were predicted to be *L. sanfranciscensis*. To confirm the identity of the bacterial colonies, all 53 samples of gDNA were analyzed using 16S rRNA gene sequencing. The 34 isolates that had detectable bands in the species-specific PCR assay were identified as *L. sanfranciscensis* with high homology values. In addition, colonies that had no detectable bands in the species-specific PCR assay were identified as other LAB including *Lactobacillus curvatus, Lactobacillus brevis, Lactobacillus plantarum*, and *Lactobacillus sakei* (Table 2). These results support that the primer set developed in this study can rapidly and specifically detect *L. sanfranciscensis*. Additionally, 11 *L. sanfranciscensis* isolates were detected by species-specific PCR and thus, a total of 45 isolates were used for further genotyping and phenotyping analyses.

3.3. Multiplex-RAPD PCR analysis

In order to study the diversity of the 45 *L. sanfranciscensis* isolates from Korean sourdough, both genotypic and phenotypic analyses were conducted. First, the multiplex-RAPD analysis, which uses two oligonucleotide primers in a single reaction, was performed. This method is considered appropriate for bacterial genotyping based on its discriminating power (Simpson's Index (D) value: 0.87 and 0.92, respectively) and reproducibility level (82%) (Venturi et al., 2012). Two sets of multiplex-RAPD analysis (OPL-05 + RD1 and P1 + RD1) were performed. The results were combined together to generate a

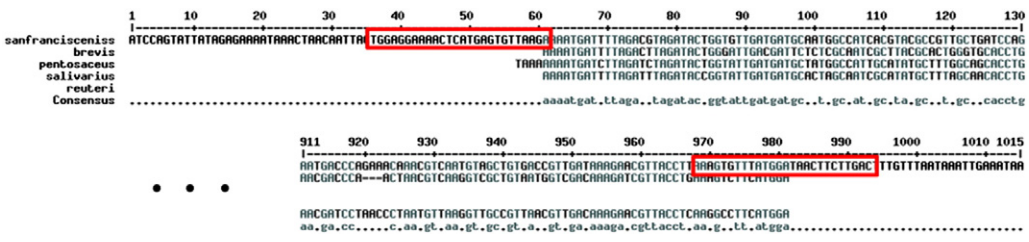

Fig. 1. The alignment of the *L. sanfranciscensis* LSA_02510 hypothetical protein gene with nucleotide sequences from other species that show sequence similarity based on the 'Discontinuous Megablast' (NCBI). The alignment was conducted based on MULTALIN (http://multalin.toulouse.inra.fr/multalin/) and intermediate sequences were omitted. Abbreviations: sanfranciscensis, *L. sanfranciscensis* TMW 1.1304; brevis, *L. brevis* KB 290; pentosaceus, *Pediococcus pentosaceus* ATCC 25745; salivarius, *Lactobacillus salivarius* UCC 118; and reuteri, *Lactobacillus reuteri* SD 2112. Species-specific primers were designed based on the boxes above.

Fig. 2. Results of the species-specific PCR assay using *L. sanfranciscensis* and other reference strains. Lane 1: DNA ladder (1 kb), lane 2: *L. sanfranciscensis* ATCC 27651, lane 3: *L. sanfranciscensis* JHS 55, lane 4: *L. sanfranciscensis* JHS 101, lane 5: *L. plantarum* ATCC 8014, lane 6: *L. brevis* DSM 6235, lane 7: *L. paracasei* ATCC 25302, lane 8: *L. sakei* subsp. *sakei* ATCC 31063, lane 9: *L. casei* ATCC 393, lane 10: *L. lactis* subsp. *lactis* ATCC 19435, lane 11: *P. pentosaceus* ATCC 33314, lane 12: *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 9135, lane 13: *L. crustorum* KACC 16344, lane 14: *Leuconostoc citreum* ATCC 49370, lane 15: *L. curvatus* ATCC 25601, lane 16: *L. paralimentarius* JCM 10415, and lane 17: *L. buchneri* ATCC 4005.

unique clustering profile as shown in Fig. 3. The 45 *L. sanfranciscensis* isolates from Korean sourdough were divided into 5 clusters with the similarity cutoff value set to 75%. The Shannon Index (H) value, which refers to the biodiversity of sourdoughs (Kaneko et al., 1977), was 1.3. This value was relatively high compared to previous studies (sourdough B: 1.51, sourdough E: 1.06, sourdough G: 0.62) (Venturi et al., 2012). Because the isolates of *L. sanfranciscensis* were divided into several groups in a single Korean sourdough, strain level characterization was necessary to obtain more biochemical information for each cluster.

3.4. VITEK2 Compact analysis

The phenotypic diversity of the *L. sanfranciscensis* isolates was investigated using the VITEK2 Compact system. The GP card of the VITEK2 system includes biochemical tests to determine carbohydrate usage, enzyme activity, and resistance to certain compounds that can be used to identify gram-positive, non-spore-forming bacteria (Pincus, 2006). Although it is primarily used to identify cocci (Wallet et al., 2005), we used this system to differentiate *L. sanfranciscensis* strains (Crowley et al., 2012). As shown in Table 3, some phenotypes were similar within isolates: over 90% of the strains tested reacted with D-amygdalin and alanine arylamidase, and 1% reacted with leucine arylamidase, tyrosine arylamidase, novobiocin, arginine dihydrolase 2, and optochin. However, there was higher intra-species diversity in phenotypes of substrate utilization among the isolates of *L. sanfranciscensis*.

Clustering analyses based on the simple matching coefficient and UPGMA methods identified strains showing diverse reaction patterns that divided into 7 clusters with the cutoff value set at 92% similarity (Fig. 4). The discriminatory power of the VITEK2 Compact system was estimated to be 0.81 based on Simpson's Index (D). This suggests that this system could be used as an alternative method for differentiating phenotypes at the strain level.

Subtyping of *L. sanfranciscensis* at the strain level using phenotypic characterization has been done previously. One study used the API 50CHL (API system, BioMerieux, France) to compare the fermentation capability of a number of strains and identified 2 clusters (Foschino et al., 2001). Another study used the Biolog system (Biolog, Inc., Hayward, CA, USA) to compare the carbon usage pattern of *L. sanfranciscensis* strains and identified 5 groups based on a cutoff of 92% similarity (De Angelis et al., 2007). The system used in this study tested not only the carbon usage, but also the enzyme activity and resistance to certain compounds. Thus, it could be noticed that the VITEK2 Compact system is able to differentiate strains and provide a high clustering result.

The diversity of *L. sanfranciscensis* strains in traditional Korean sourdough could be derived from the broad microbial spectrum in the nuruk used in this study (Jung-Hun, 2000; Song et al., 2013; Yoon et al., 2012). Nuruk is a traditional natural fermentation starter that possesses a diverse microbial population, since various microorganisms are incorporated from the surrounding environment during its production. In addition to sourdough bread, nuruk has been used as a starter to provide a microbial source for alcoholic fermentation of Makgeolli, a Korean traditional alcoholic beverage. The rich microbial diversity in Makgeolli was shown to be from nuruk using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR–DGGE) (Kim et al., 2010) and next-generation sequencing analyses (Jung et al., 2012).

Prior to choosing sourdough starters, each species should be differentiated at a strain level since the desired properties of the dough and bread are strain-dependent. For example, Robert et al. (2006) demonstrated the differences in acidification rate and soluble carbohydrate availability when using *L. plantarum* and *Leuconostoc* spp. as starter strains. In addition, enzyme activities including β-glucosidase, phytase, and urease of *Lactobacillus* starters were different at a strain level (Zotta et al., 2007). These studies show the importance of subtyping strains when selecting starters. For industrial application, following differentiation studies, the starters for making sourdough bread in each cluster should be evaluated for several parameters such as carbon utilization, organic acid production, antimicrobial activity, and exopolysaccharide formation against each strain.

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Conflict of interests

Jin Soo Hur and Sangmin Shim are employed by SPC Group and the other authors declare no conflict of interest.

Table 2
Summary of the species-specific PCR and identification of sourdough isolates.

16S rRNA gene Sequencing results	Identity (%)	Isolate no.	PCR amplification
L. sanfranciscensis ATCC 27651	100	101, 107, 125, 129, 133, 141, 145, 186, 196, 207, 210, 217, 220, 225	Yes
	99	105, 110, 113, 115, 121, 123, 124, 131, 135, 137, 148, 161, 166, 170, 179, 195, 223, 235, 236, 245	Yes
L. curvatus ATCC 25601	99	104, 109, 112, 116, 120, 122, 127, 130, 159,206,218	No
L. brevis ATCC 367	99	111, 118, 149	No
L. plantarum ATCC 13649	100	119, 128, 143, 158	No
L. sakei subsp. *sakei* ATCC 15521	99	219	No
P. pentosaceus ATCC 25745	99	224	No

ATCC — American Type Culture Collection, Rockville, Maryland, USA.

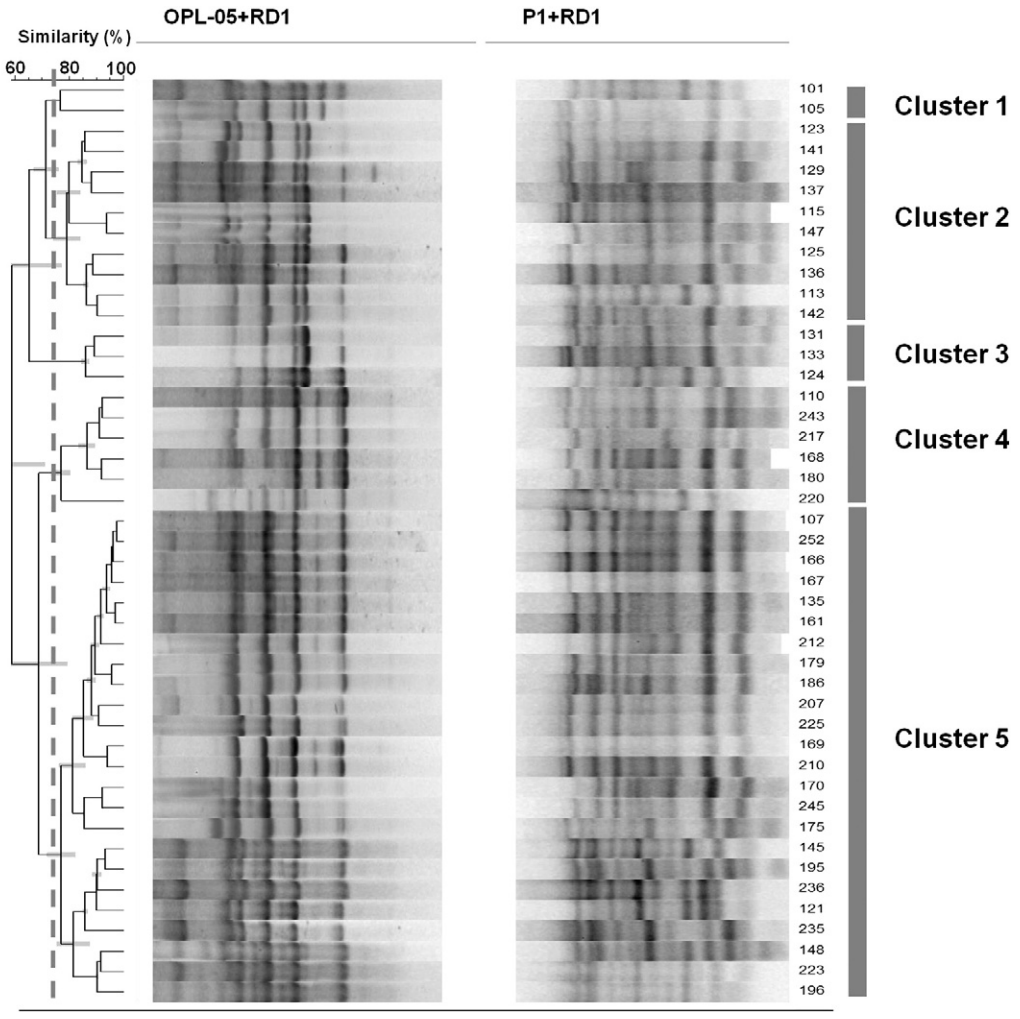

Fig. 3. Dendrogram derived from the combined RAPD-PCR analysis of *L. sanfranciscensis* isolates from Korean sourdough using the primers OPL-05 + RD1 and P1 + RD1. The clustering was based on the Dice coefficient and unweighted pair group with arithmetic average (UPGMA) analyses.

Table 3
Usage of the test substrates on GP card by *L. sanfranciscensis* isolates.

Well No.	Test	Substrate usage[a] (%)	Well No.	Test	Substrate usage (%)
2	D-amygdalin	90	32	Polymixin B resistance	0
4	Phosphatidylinositol phospholipase C	0	37	D-galactose	29
5	D-xylose	24	38	D-ribose	0
8	Arginine dihydrolase 1	0	39	L-lactate alkalinization	0
9	Beta-galactosidase	0	42	Lactose	13
11	Alpha-glucosidase	0	44	N-acetyl-D-glucosamine	47
13	Ala-Phe-Pro arylamidase	11	45	D-maltose	62
14	Cyclodextrin	58	46	Bacitracin resistance	0
15	L-aspartate arylamidase	0	47	Novobiocin resistance	1
16	Beta galactopyranosidase	0	50	Growth in 6.5% NaCl	0
17	Alpha-mannosidase	0	52	D-mannitol	73
19	Phosphatase	0	53	D-mannose	38
20	Leucine arylamidase	1	54	Methyl-β-D-glucopyranoside	69
23	L-prolinearylamidase	0	56	Pullulan	64
24	Beta glucuronidase	0	57	D-raffinose	67
25	Alpha-galactosidase	0	58	O/129 resistance	0
26	L-pyrrolidonyl-arylamidase	0	59	Salicin	64
27	Beta-glucuronidase	0	60	Saccharose/sucrose	60
28	Alanine arylamidase	100	62	D-trehalose	60
29	Tyrosine arylamidase	1	63	Arginine dihydrolase 2	1
30	D-sorbitol	31	64	Optochinresistance	1

[a] Percentage of isolates utilizing substrates among total isolates.

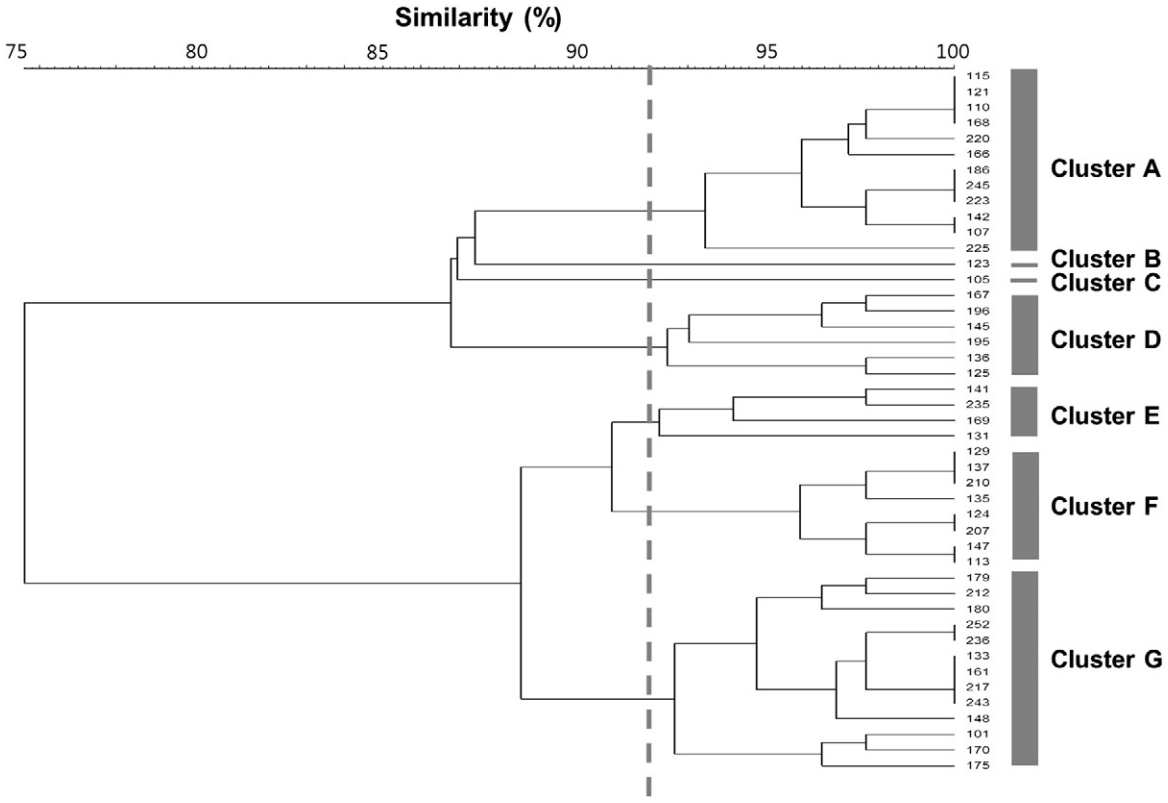

Fig. 4. Results of the VITEK2 Compact phenotypic analysis and UPGMA cluster analysis for *L. sanfranciscensis* isolates from Korean sourdough. The cluster analysis was conducted using a simple matching coefficient.

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