Lactobacillus kimchiensis sp. nov., isolated from a fermented food

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A novel bacterium was isolated from a traditional fermented food, kimchi. The morphology, physiology, biochemical properties and 16S rRNA gene sequence of strain L133^T were studied. Strain L133^T was Gram-reaction-positive, catalase-negative and homofermentative, with rod-shaped cells that formed cream colonies. Cells grew in the presence of 0–5 % (w/v) NaCl (optimum, 1–2 %), at pH 5.0–9.0 (optimum, pH 7.0–8.0) and at 15–37 °C (optimum, 25 °C). Comparative 16S rRNA gene and *pheS* sequence analysis of strain L133^T indicated that the strain belonged to the genus *Lactobacillus*. The major fatty acids were identified as $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{18:0}$, and the cell wall contained peptidoglycan of the L-Lys–D-Asp type. DNA–DNA relatedness values between strain L133^T and related species were below 11 ± 0.4 %. The DNA G+C content of strain L133^T was 35.7 mol%. Analysis of 16S rRNA gene sequences, as well as physiological and biochemical tests, identified genotypic and phenotypic differences between strain L133^T and other species of the genus *Lactobacillus*. Based on these analyses, strain L133^T is proposed to be a novel species of the genus *Lactobacillus*, named *Lactobacillus kimchiensis*. The type strain is L133^T (=KACC 15533^T=JCM 17702^T=DSM 24716^T).

Kimchi is a traditional Korean fermented food made of various vegetables and spices (red pepper, garlic, ginger, *etc.*). This food includes a high number of lactic acid bacteria (LAB) ($10^{8–9}$ c.f.u. ml^{-1}) (Park & Kim, 2010) and organic acids (*e.g.*, lactic, acetic, succinic and propionic acids) during the fermentation process. Fermentation is known to be strongly affected by environmental factors, such as the temperature, salt concentration, ingredients and fermentation period, and the microflora, including LAB (Park *et al.*, 2010). LAB have important effects on the taste of kimchi (Kim *et al.*, 1986) and many of these LAB isolates possess antimicrobial activity and other useful properties (Lee *et al.*, 1997; Mheen & Kwon, 1984; So & Kim, 1995). Thus, LAB have been extensively studied to acquire a better understanding of their roles in the kimchi fermentation process, with the aim of manipulating the fermentation process (Bae *et al.*, 2005). The current study of LAB presents the morphological, biochemical and molecular characterization of novel LAB strain L133^T.

Strain L133^T was isolated from kimchi using the dilution-plating method with lactobacilli MRS Agar medium (MRSA; BBL) at 25 °C for 3 days. Cell morphology was inspected by light microscopy (Eclipse 50*i*; Nikon). Salt tolerance and requirements were investigated using lactobacilli MRS broth (MRS; BBL) medium without NaCl, and supplementing with NaCl at various concentrations [0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 % (w/v)]. Growth temperature (4, 15, 25, 30, 37 and 45 °C) and pH (pH 3.0–11.0 at intervals of 1.0 pH) were tested using MRS broth (MRS; BBL). Different pH values were adjusted using 10 mM MES (for pH 4, 5 and 6), 10 mM TAPS (for pH 7, 8 and 9) or 10 mM Na_2HPO_4 (for pH 10 and 11). Results were recorded after 72 h. Growth occurred between pH 5.0 and 9.0 in MRS broth (optimum, pH 7.0–8.0), with a NaCl range of 0–5 % (optimum, 1–2 %) and a temperature of 15–37 °C (optimum, 25 °C). To analyse the physiological and morphological characteristics, strain L133^T was routinely cultivated on MRS agar or in MRS broth medium at the optimum temperature and NaCl concentration. The Gram reaction was determined using a 3 % KOH method (Buck, 1982). Catalase and oxidase tests were performed with 3 % H_2O_2 and oxidase reagent (bioMérieux), respectively. Carbohydrate fermentation tests were conducted using the API 50CHL strip with

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Abbreviations: LAB, lactic acid bacteria; ME, minimum-evolution; MP, maximum-parsimony; NJ, neighbour-joining; TAP-PCR, triplicate arbitrarily primed-PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *pheS* sequences of strain L133^T are HQ906500 and JQ713530, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.

API AUX medium (bioMérieux), according to the manufacturer's instructions. Gas production from glucose was analysed as described previously (Davis, 1955). The formation of lactate isomers in the fermented broth was determined enzymically using the UV method (R-biopharm). Strain L133^T colonies grown on MRSA medium were cream, circular, smooth, opaque and 1.0–2.0 mm in diameter after incubation for 3 days. Cells were Gram-reaction-positive, catalase-negative rods, which measured approximately 1.0–5.0 μm in length. No gas was released and the ratio of L- to D-lactate was 80 : 20. A detailed species description is presented below and Table 1 provides a phenotypic comparison of strain L133^T and closely related species of the genus *Lactobacillus*, based on 16S rRNA gene sequence similarity. The reference strains used were *Lactobacillus crustorum* LMG 23699^T (Scheirlinck *et al.*, 2007), *Lactobacillus farciminis* DSM 20184^T (Reuter, 1983), *Lactobacillus mindensis* DSM 14500^T (Ehrmann *et al.*, 2003), *Lactobacillus bobalius* 203^T (Mañes-Lázaro *et al.*, 2008), *Lactobacillus nantensis* LP33^T (Valcheva *et al.*, 2006) and *Lactobacillus paralimentarius* TB 1^T (Cai *et al.*, 1999).

Genomic DNA was extracted using a DNA extraction kit (iNtRON Biotechnology). The 16S rRNA gene was amplified with a PCR Pre-Mix and two universal primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTGTTACG-ACTT-3′). The PCR product was purified with a PCR purification kit (Qiagen) and sequencing was performed as described previously (Roh *et al.*, 2008). SeqMan software (DNASTAR) was used to assemble the almost full-length 16S rRNA gene sequences (1436 bp). Sequence similarities were determined using the EzTaxon server (Chun *et al.*, 2007) to locate phylogenetic neighbours. The phylogenetic relationships of representative species of the genus *Lactobacillus* were determined using the MEGA4 software program (Tamura *et al.*, 2007). Phylogenetic tree construction was performed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Kluge & Farris, 1969) methods. The topologies of the trees were determined by compiling a consensus tree based on 1000 randomly generated trees (Felsenstein, 1985). Based on the 16S rRNA gene sequence similarities, strain L133^T was most closely related to *L. crustorum* LMG 23699^T (98.3 %), *L. farciminis* DSM 20184^T (98.0 %), *L. mindensis* DSM 14500^T (97.9 %), *L. kimchii* MT-1077^T (97.7 %), *L. bobalius* 203^T (97.6 %), *L. nantensis* LP33^T (97.6 %) and *L. paralimentarius* TB 1^T (96.4 %). In the phylogenetic relationship, strain L133^T was closely associated with the *Lactobacillus* cluster, sharing a branching point with the species *L. crustorum* LMG 23699^T, *L. farciminis* DSM 20184^T, *L. nantensis* LP33^T and *L. mindensis* DSM 14500^T (Fig. 1 and Fig. S1 available in IJSEM Online). The phylogenetic status of strain L133^T was also investigated using *pheS* gene sequence analysis (Naser *et al.*, 2007). Phylogenetic analysis using the neighbour-joining (Saitou & Nei, 1987) method based on *pheS* gene sequences was performed similarly to the 16S rRNA gene sequence analysis above. Comparisons of the *pheS* gene sequences were made using pairwise alignment on the EzTaxon server (Chun *et al.*, 2007) to locate phylogenetic neighbours. Phylogenetic analysis based on *pheS* gene sequences indicated that strain L133^T was a member of the *Lactobacillus alimentarius* group (Fig. S2) (Naser *et al.*, 2007) and was closely related to *L. nantensis* LP33^T (87.2 %), *L. kimchii* MT-1077^T (85.9 %), *L. farciminis* DSM 20184^T (85.5 %), *L. paralimentarius* TB 1^T (84.6 %), *L. crustorum* LMG 23699^T (84.2 %) and *L. mindensis* DSM 14500^T (83.5 %). An interspecies gap was provided by the *pheS* gene sequence analysis, the gap is normally above 10 % divergence (Naser *et al.*, 2007).

The genomic DNA G+C content was determined by a fluorimetric method with SYBR Green I using a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). For the analysis, strain L133^T was grown on MRSA for 3 days at 25 °C. Calibration was conducted using genomic DNA from completely sequenced *Escherichia coli* K-12, *Halorubrum lacusprofundi* DSM 5036^T and *Ruminococcus obeum* ATCC 29174^T. The G+C content of genomic DNA for species in the genus *Lactobacillus* ranged from 32 mol% to 59.2 mol% (Cai *et al.*, 2012). The DNA G+C content of

Table 1. Differential phenotypic characteristics of strain L133^T and closely related members of the genus *Lactobacillus*

Strains: 1, L133^T; 2, *L. crustorum* LMG 23699^T; 3, *L. farciminis* DSM 20184^T; 4, *L. mindensis* DSM 14500^T; 5, *L. bobalius* 203^T. All data from this study. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5
Growth in the presence of 10 % NaCl	−	−	+	−	−
DNA G+C content (mol%)*	35.7	35–36	35	37.5	34.03±0.7
Acid production from:					
D-Ribose	−	−	−	−	+
D-Galactose	+	+	+	−	W
Methyl α-D-glucoside	−	−	+	−	−
Amygdalin	W	+	−	−	+
Arbutin	W	+	+	−	+
Salicin	+	+	+	−	+
Cellobiose	+	+	+	−	+
Maltose	+	+	W	−	+
Lactose	+	+	+	−	−
Sucrose	+	−	+	−	+
Trehalose	+	+	+	−	+
Melezitose	−	−	−	−	+
Gentiobiose	W	W	−	−	W
Turanose	+	−	−	−	−
D-Tagatose	+	+	+	−	−
Gluconate	−	−	−	−	W

*Data for reference species from Scheirlinck *et al.* (2007), Reuter (1983), Ehrmann *et al.* (2003) and Mañes-Lázaro *et al.* (2008).

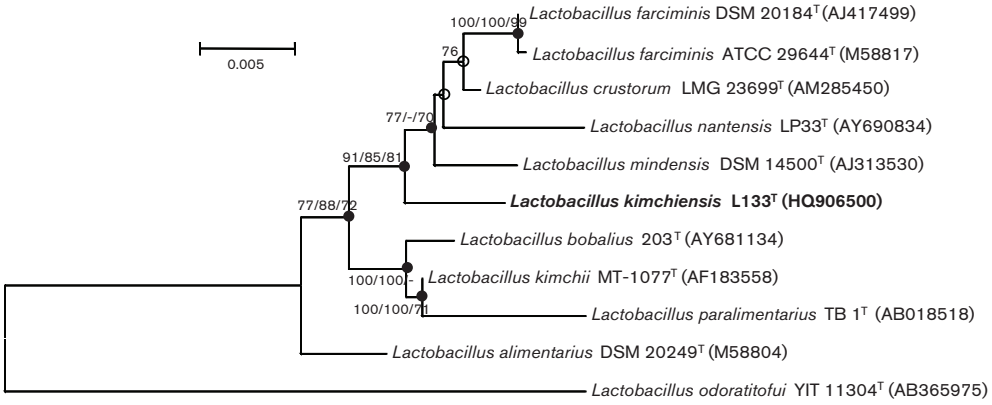

Fig. 1. Phylogenetic consensus tree showing the phylogenetic position of the novel strain based on 16S rRNA gene sequences using the neighbour-joining (NJ) algorithm. Trees were also constructed using the minimum-evolution (ME) and maximum-parsimony (MP) algorithms. GenBank accession numbers are shown in parentheses. Filled circles and open circles indicate generic branches that were also identified using both the ME and MP algorithms, and either the ME or MP algorithm, respectively. Numbers at the nodes indicate bootstrap values (>70 %) calculated on the basis of NJ/ME/MP probabilities and expressed as percentages of 1000 replicates. Bar, 0.005 accumulated changes per nucleotide.

strain L133^T was 35.7 mol%, which was in the range for members of the genus *Lactobacillus*. DNA–DNA hybridization was performed using genome-probing microarrays (Bae *et al.*, 2005; Chang *et al.*, 2008). DNA–DNA relatedness values of strain L133^T (as the target strain) with *L. bobalius* 203^T, *L. crustorum* LMG 23699^T, *L. farciminis* DSM 20184^T and *L. mindensis* DSM 14500^T were 6±0.4 %, 11±0.4 %, 5±0.2 % and 7±0.5 %, respectively, and for the reciprocal experiments (strain L133^T as the probe) were 14±0.7 %, 15±1.4 % and 14±0.6 % with *L. bobalius* 203^T, *L. crustorum* LMG 23699^T and *L. farciminis* DSM 20184^T, respectively (Table S1). The genomic distinctiveness (Wayne *et al.*, 1987) is sufficient for classification of this strain as a distinct species.

Quantitative analysis of cellular fatty acids was conducted for strain L133^T and reference species using cells grown on MRSA for 3 days at 28 °C. Cellular fatty acids were extracted according to the Sherlock Microbial Identification System (MIDI), analysed by gas chromatography (model 6890; Hewlett Packard) and identified using the Microbial Identification software package. The cellular fatty acid composition of strain L133^T was as follows: $C_{18:1}\omega9c$ (49.9 %), $C_{16:0}$ (14.6 %), $C_{18:0}$ (12.7 %), summed feature 7 (unknown constituent with equivalent chain length of 18.846, $C_{19:1}\omega6c$ and $C_{19:0}$ cyclo$\omega10c$; 7.2 %), $C_{18:1}\omega7c$ (7.1 %), anteiso-$C_{15:0}$ (3.3 %), $C_{14:0}$ (2.6 %), iso-$C_{16:0}$ (1.7 %) and iso-$C_{14:0}$ (1.0 %). This profile, where $C_{18:1}\omega9c$ is the major fatty acid, is characteristic of members of the genus *Lactobacillus*. The cellular fatty acid profile of strain L133^T was qualitatively similar to that of the reference species (Table 2). The amino acid composition of the cell wall hydrolysate was determined using one-dimensional TLC on

Table 2. Fatty acid composition (%) of strain L133^T and closely related members of the genus *Lactobacillus*

Strains: 1, L133^T; 2, *L. crustorum* LMG 23699^T; 3, *L. farciminis* DSM 20184^T; 4, *L. mindensis* DSM 14500^T; 5, *L. bobalius* 203^T. All data from this study. Values are percentages of the total fatty acids. Fatty acids that represented <0.5 % in all species were omitted. TR, trace amount (<0.5 %); −, not detected.

Fatty acid	1	2	3	4	5
Saturated acids					
$C_{12:0}$	−	TR	TR	−	TR
$C_{14:0}$	2.6	1.0	1.3	1.3	1.1
$C_{16:0}$	14.6	20.9	20.5	21.5	19.8
$C_{18:0}$	12.7	2.2	1.9	2.1	2.1
Unsaturated acids					
$C_{18:1}\omega7c$	7.1	−	−	−	−
$C_{18:1}\omega9c$	49.9	34.4	46.7	59.3	45.7
Branched acids					
iso-$C_{14:0}$	1.0	−	−	−	−
anteiso-$C_{15:0}$	3.3	−	−	−	−
iso-$C_{16:0}$	1.7	−	−	−	−
iso-$C_{19:1}$	−	−	−	−	TR
Summed features*					
3	−	2.0	1.8	1.9	1.4
7	7.2	24.0	35.2	23.7	10.5
8	−	4.7	4.3	3.8	3.8

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega6c$/$C_{16:1}\omega7c$; summed feature 7 consisted of unknown constituent with equivalent chain length of 18.846/$C_{19:1}\omega6c$/$C_{19:0}$ cyclo $\omega10c$; summed feature 8 consisted of $C_{18:1}\omega6c$/$C_{18:1}\omega7c$.

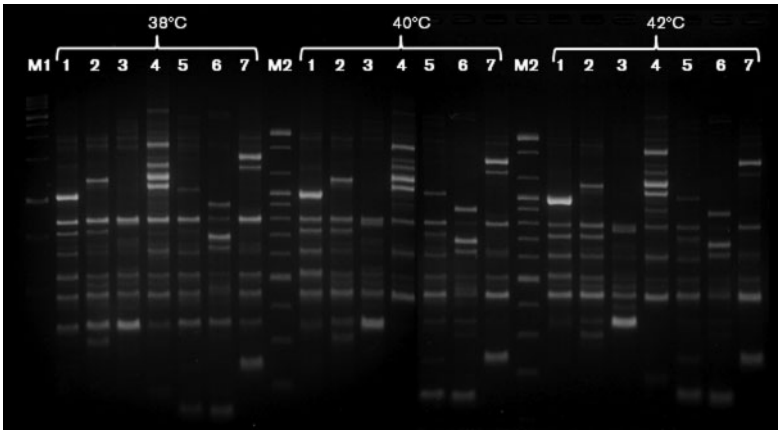

Fig. 2. TAP-PCR-generated fingerprints of strain L133^T and closely related members of the genus *Lactobacillus*. Lanes: 1, strain L133^T; 2, *L. crustorum* LMG 23699^T (Scheirlinck *et al.* 2007); 3, *L. farciminis* DSM 20184^T (Reuter 1983); 4, *L. mindensis* DSM 14500^T (Ehrmann *et al.* 2003); 5, *L. bobalius* 203^T (Mañes-Lázaro *et al.* 2008); 6, *L. nantensis* LP33^T (Valcheva *et al.*, 2006); 7, *L. paralimentarius* TB 1^T (Cai *et al.*, 2012); M1, 1 kb ladder; M2, 100 bp ladder (Bioneer).

cellulose sheets (Bousfield *et al.*, 1985). The diagnostic amino acids for the cell wall of strain L133^T were L-lysine and D-aspartic acid, indicating an A4a L-Lys–D-Asp peptidoglycan type. This result was consistent with those found for other members of the genus *Lactobacillus*, supporting the affiliation of strain L133^T to this genus.

Triplicate arbitrarily primed-PCR (TAP-PCR) was used for the genomic-based differentiation of strain L133^T from the reference species. This fingerprinting methodology uses a primer specific for a conserved region in the 16S rRNA gene and it was evaluated for the molecular fingerprinting of a wide range of LAB genera (Cusick & O'Sullivan, 2000). The conditions for PCR amplification were as follows: initial denaturation at 92 °C for 2 min; 40 cycles of denaturation at 92 °C for 30 s, annealing at 38, 40 or 42 °C for 1 min and extension at 68 °C for 90s; final extension at 68 °C for 10 min (Cusick & O'Sullivan, 2000). All patterns generated were unique and capable of discriminating all strains tested. Strain L133^T could be differentiated from the reference strains using TAP-PCR fingerprinting profiles (Fig. 2).

Based on the results of phenotypic, genotypic and phylogenetic studies, it is concluded that strain L133^T is a novel species of the genus *Lactobacillus* and the name *Lactobacillus kimchiensis* sp. nov. is proposed.

Description of *Lactobacillus kimchiensis* sp. nov.

Lactobacillus kimchiensis (Kim.chi.en′sis. N.L. n. *kimchium* kimchi, a type of traditional Korean fermented food; L. masc. suff. *-ensis* suffix used with the sense of belonging to; N.L. masc. adj. *kimchiensis* belonging to kimchi, isolated from kimchi).

Cells are Gram-reaction-positive, catalase- and oxidase-negative rods, measuring approximately 1.0–5.0 μm in length. Growth occurs at 15–37 °C (optimum, 25 °C), at pH 5.0–9.0 (optimum, pH 7.0–8.0) and with 0–5 % (w/v) NaCl (optimum, 1–2 %). Gas production from glucose is not detected, so the fermentation type of strain L133^T is homofermentation. The ratio of L- to D-lactate is 80 : 20. Acid is produced from D-galactose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, turanose, D-tagatose, amygdalin, arbutin and gentiobiose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The major cellular fatty acids are $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{18:0}$. The cell wall contains peptidoglycan of the L-Lys–D-Asp type.

The type strain, L133^T (=KACC 15533^T=JCM 17702^T=DSM 24716^T), was isolated from a traditional fermented food in Korea. The DNA G+C content of the type strain L133^T is 35.7 mol%.

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