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TAXONOMIC DESCRIPTION

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Lactococcus kimchii sp. nov., a new lactic acid bacterium isolated from kimchi

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Abstract

A coccus strain designated S-13^T was isolated from commercial baechu-kimchi in Korea. Comparison of the 16S rRNA gene sequence indicated that strain S-13^T had the highest similarity to *Lactococcus taiwanensis* 0905C15^T (97.9%), *Lactococcus lactis* subsp. *tructae* L105^T (97.6%), *Lactococcus lactis* subsp. *cremoris* NCDO 607^T (97.5%), *Lactococcus lactis* subsp. *hordniae* NBRC 100931^T (97.2%), and *Lactococcus lactis* subsp. *lactis* JCM 5805^T (97.2%). The detailed phylogenetic analyses based on the 16S rRNA, *rpoB* and *recA* genes indicated that S-13^T was separated from the other species and subspecies in the genus *Lactococcus*. The DNA–DNA relatedness between S-13^T and closely related type strains, such as *L. taiwanensis* 0905C15^T, *L. lactis* subsp. *tructae* L105^T, *L. lactis* subsp. *cremoris* NCDO 607^T, *L. lactis* subsp. *hordniae* NBRC 100931^T, and *L. lactis* subsp. *lactis* JCM 5805^T was 25.6, 20.4, 25.1, 20.2 and 21.7%, respectively. The major fatty acids were $C_{16:0}$, cyclo-$C_{19:0}\omega 8c$ and $C_{14:0}$. The DNA G+C content of S-13^T was 39.4 mol%. From the results of the phenotypic characteristics and chemotaxonomic analysis, it was concluded that strain S-13^T represents a novel species in the genus *Lactococcus* for which the name *Lactococcus kimchii* sp. nov. (=KCTC 21096^T=NBRC 113348^T) is proposed.

Lactic acid bacteria are important in the food industry because they have been used as starter cultures for pickles, beverages, cheese, bread, soy sauce, and many animal feeds. *Lactococcus lactis* is an organism of substantial economic importance used widely in the production of fermented dairy products such as cheese and yogurt [1]. Fermented foods have been consumed in Korea for more than 2000 years [2, 3]. Kimchi is one example of fermented vegetables which is fermented mainly by lactic acid bacteria. Baechu kimchi is made from brined Chinese cabbage (baechu) together with the addition of fermented fish or shrimp sauce and supplementary vegetables such as radishes, garlic, red pepper powder, cucumbers, and green onions. The genus *Lactococcus* is a group of lactic acid bacteria in the family *Streptococcaceae* that is catalase-negative and has homofermentative metabolism. Many species of the genus *Lactococcus* have been isolated from milk, but several species such as *Lactococcus plantarum, Lactococcus formosensis*, *Lactococcus fujiensis,* and *Lactococcus taiwanensis* have plant origins, too. *L. formosensis* was isolated from fermented broccoli stems [4]. In this study, one strain, designated S-13^T, isolated from Korean kimchi was examined by a polyphasic taxonomic approach to determine its exact taxonomic position. The result indicated that the isolate represents a novel species in the genus *Lactococcus*.

The cabbage kimchi was bought randomly at a market in Seoul during the winter season in January. Ten grams of cabbage kimchi were homogenized in 1000 ml of distilled water and then filtered through Whatman number 1 paper (Whatman). The filtrate was plated on de Man, Rogosa and Sharpe agar (MRS; Difco) by a serial dilution procedure and incubated at 25 °C with the BD Gaspack in a gas tight jar. The colonies formed were picked out and purified by the streaking method using the same culture conditions. Strain S-13^T was preserved in 10% skim milk stock at −80 °C.

The 16S rRNA gene of S-13^T was amplified using the universal bacterial primer pairs of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACT

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Keywords: baechu kimchi; fermented vegetable; lactic acid bacteria.
Abbreviations: ML, maximum-likelihood; MP, maximum parsimony; NJ, neighbour-joining.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence is MG576055. The sequences of the *recA* and *rpoB* genes were obtained from the whole genome of S-13^T which was deposited with the accession numbers SDAK01000001, SDAK01000002, SDAK01000003 and SDAK01000004.
One supplementary figure is available with the online version of this article.

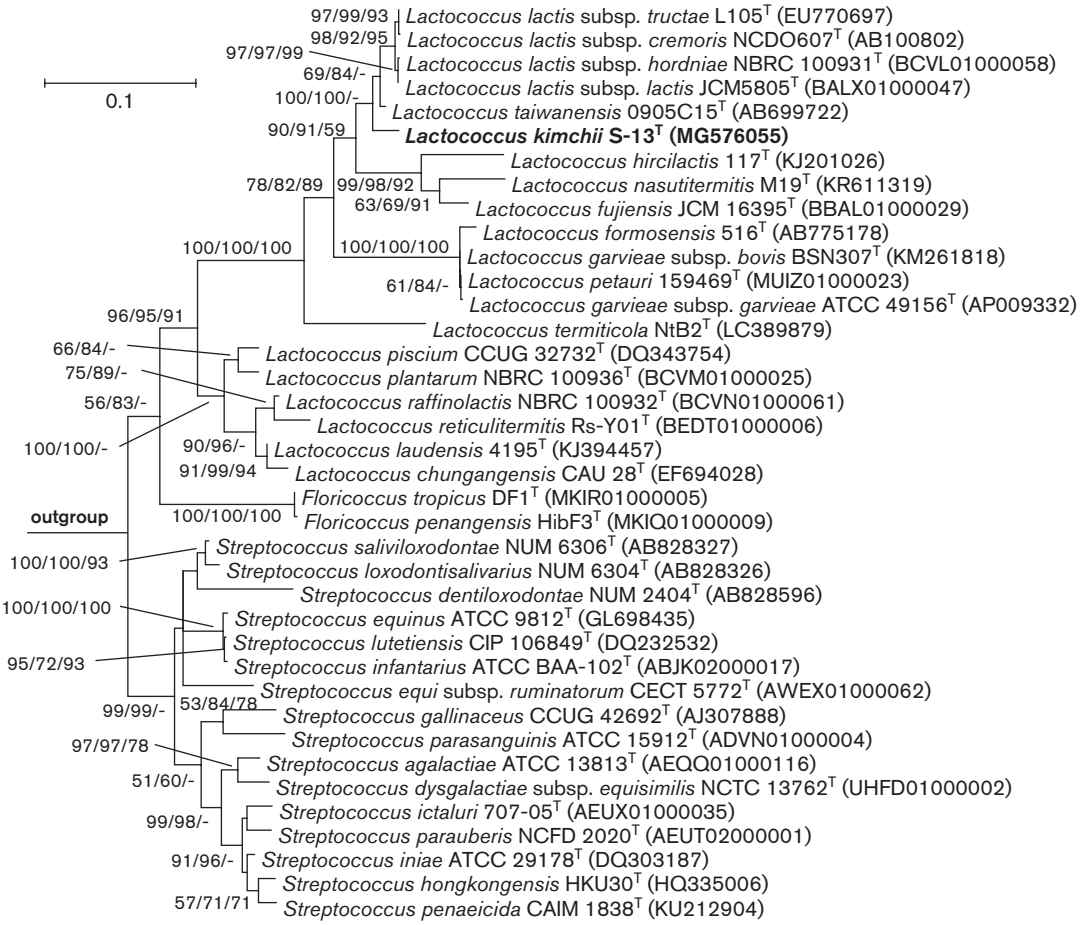

Fig. 1. Maximum-likelihood tree of Lactococcus *kimchii* S-13^T and other related species based on an alignment of 1379 nucleotides of 16S rRNA gene sequences. Bootstrap values (>50%) in the order of ML/NJ/MP are shown at the branch points based on 1000 replications. *Bacillus subtilis* DSM10^T (AJ276351) was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

T-3′). Then, the gene was sequenced using the universal bacterial primers and two additional primers: 518F (518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 518R (5′-GTATTACCGCGGCTG G-3′). The sequencing of the gene was carried out in an ABI3730XL automated sequencer (Applied Biosystems) by BioFact (Daejeon, Republic of Korea). The 16S rRNA gene sequence was compared by using the EzBiocloud sever [5]. All sequences of the related strains retrieved from EzBiocloud were aligned and edited with the BioEdit [6] and CLUSTAL X software [7]. The phylogenetic trees were reconstructed by using neighbour-joining (NJ) [8], maximum-likelihood (ML) [9], and maximum parsimony (MP) [10] methods in the MEGA7 program [11]. Tree topologies were evaluated by bootstrap analysis based on 1000 replications [12]. On the basis of the 16S rRNA similarity values and the clustering on the phylogenetic tree, *Lactococcus taiwanensis* NBRC 109049^T, *Lactococcus lactis* subsp. *tructae* DSM 21502^T, *Lactococcus lactis* subsp. *cremoris* KCTC 3619^T, *Lactococcus lactis* subsp. *hordniae* KCTC 3768^T, and *Lactococcus lactis* subsp. *lactis* KCTC 3769^T were chosen as reference strains.

The genomic DNA of S-13^T and the reference strains was extracted from cells grown on tryptic soy agar (TSA) at 30 °C for 48 h with a NucleoSpin microbial DNA kit (Macherey-Nagel) and followed by purification using the Qiagen Genomic DNA extraction kit according to the manufacturer's instructions (Qiagen). Whole-genome sequencing was performed by Macrogen (Seoul, Republic of Korea). A library of 20 kb SMRTbell was constructed from the high molecular-weight genomic DNA (15 µg). The library was sequenced using the PacBio RS II version 4.0 single-molecule real-time (SMRT) sequencing technology (Pacific Bioscicence), yielding 378-fold average genome coverage. *De novo assembly* of the PacBio reads was completed with Hierarchical Genome Assembly Process 3 pipelines. The sequence was assembled into a 2,273,230 nt genome consisting of four contigs of one chromosome and three plasmids with an average DNA G+C content of 39.4 mol%. Gene annotation by NCBI Prokaryotic Genome Annotation Pipeline identified 2205 coding sequences including 58 pseudogenes, 62 tRNAs and 19 rRNAs. The complete genome was deposited in the GenBank/

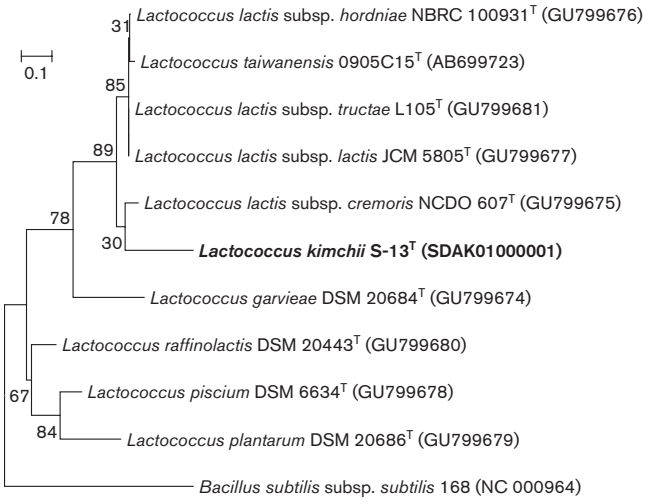

Fig. 2. Maximum-likelihood tree of Lactococcus *kimchii* S-13^T and other related species based on an alignment of the partial sequences of the *recA* gene (282 bp). Bootstrap values (>50%) are shown at the branch points based on 300 replications. *Bacillus subtilis* subsp. *subtilis* 168 (NC 000964) and *Enterococcus hirae* ATCC 9790^T (CP003504) were used as outgroups. Bar, 0.1 substitutions per nucleotide position.

EMBL/DDBJ under the accession numbers SDAK01000001, SDAK01000002, SDAK01000003 and SDAK01000004.

DNA–DNA hybridization was performed between S-13^T and *L. taiwanensis* NBRC 109049^T, *L. lactis* subsp. *tructae* DSM 21502^T, *L. lactis* subsp. *cremoris* KCTC 3619^T, *L. lactis* subsp. *hordniae* KCTC 3768^T, and *L. lactis* subsp. *lactis* KCTC 3769^T according to the method of Ezaki et al. [13]. The microplate was incubated for hybridization at 40 °C for 4 h. The quantitative DNA G+C content of S-13^T was calculated directly from the genome sequence. The *rec*A and *rpo*B nucleotide sequences of S-13^T obtained from the draft genome were used to reconstruct phylogenetic trees with the maximum-likelihood method [9]. The *rec*A and *rpo*B alignments contained 282 and 427 nucleotide positions, respectively.

Gram-staining was done with the BD Gram-stain kit according to the manufacturer's instructions. Cells of S-13^T grown overnight on TSA agar at 30 °C were used to examine the morphology using a FEI Quanta 250 FEG scanning electron microscope (FEI). The catalase activity was tested by bubble production in 3% (v/v) H_2O_2. The temperature range for growth was determined by incubating the culture on TSA agar plates at 4, 10, 15, 20, 25, 30, 37 and 40 °C. The pH range for growth was determined with two different media, MRS and TSA broth at 30 °C for 3 days. The pH values of the media

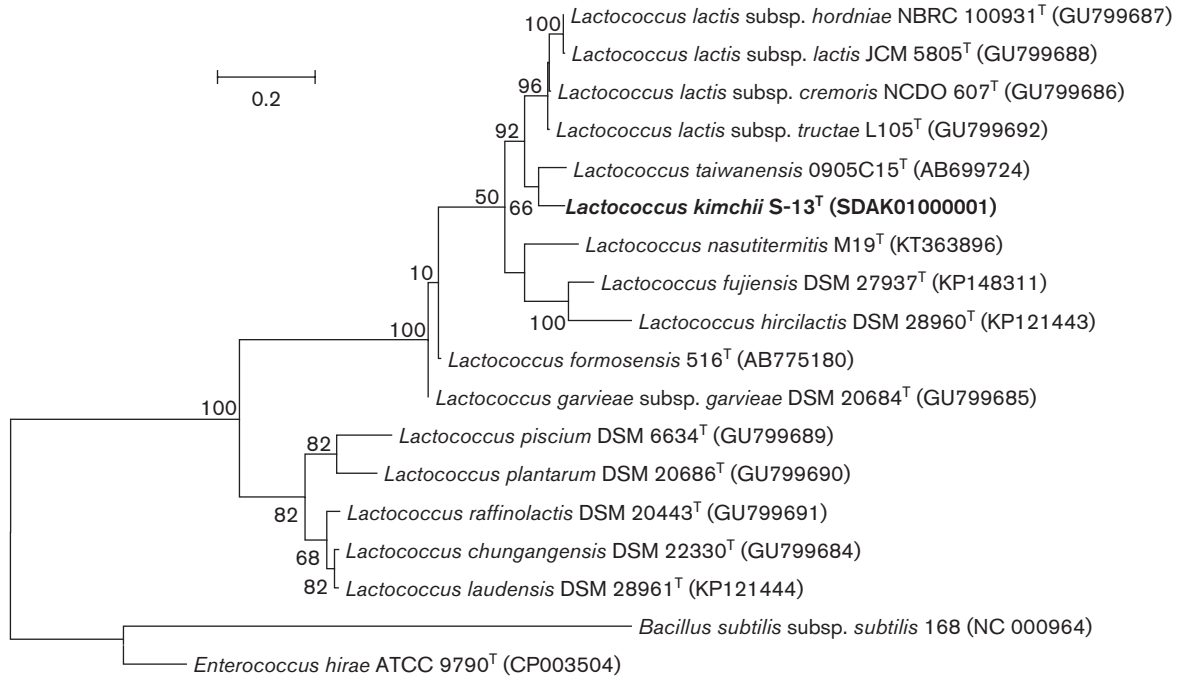

Fig. 3. Maximum-likelihood tree of Lactococcus *kimchii* S-13^T and other related species based on an alignment of the partial sequences of the *rpoB* gene (427 bp). Bootstrap values (>50%) are shown at the branch points based on 300 replications. *Bacillus subtilis* subsp. *subtilis* 168 (NC_000964) and *Enterococcus hirae* ATCC 9790^T (CP003504) were used as outgroups. Bar, 0.2 substitutions per nucleotide position.

Table 1. Comparison of the characteristics of S-13^T with those of closely related species

Strains: 1, S-13^T; 2, *Lactococcus taiwanensis* 0905C15^T; 3, *Lactococcus lactis* subsp. *tructae* L105^T; 4, *Lactococcus lactis* subsp. *cremoris* NCDO 607^T; 5, *Lactococcus lactis* subsp. *hordniae* NBRC 100931^T; 6, *Lactococcus lactis* subsp. *lactis* JCM 5805^T. +, Positive; –, negative.

Characteristics	1	2	3	4	5	6
Growth with over 4% (w/v) NaCl	–	+	+	+	+	+
Growth at 10 °C	–	–	+	+	+	–
Growth at pH 5.0	+	+	–	–	–	+
Enzyme activity						
Esterase (C4)	+	+	+	+	–	–
α-Galactosidase	–	–	+	–	–	–
α-Glucosidase	+	–	+	–	–	+
β-Glucosidase	–	–	+	–	–	–
Acid production from						
Arbutin	+	+	+	+	+	+
L-Arabinose	+	+	–	–	–	–
D-Galactose	+	+	+	–	–	+
Gentiobiose	+	+	+	–	–	+
Lactose	+	+	+	+	–	+
Maltose	+	+	+	–	–	+
D-Mannitol	+	+	+	–	–	–
Melibiose	+	–	+	–	–	–
Potassium gluconate	–	+	+	–	–	–
Sucrose	–	–	+	–	+	+
Salicin	+	+	+	–	+	+
D-Xylose	+	–	–	–	–	+
DNA G+C content (mol%)	39.4	39.6	36.0	36.5	35.5	35.9

Data for DNA G+C content were taken from a published paper by Chen *et al.* [17].

(3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) were adjusted by adding NaOH or HCl, and the pH was confirmed again after the media were autoclaved. Growth with various concentrations of NaCl (0–6.0% at increments of 1.0 %) was investigated by supplementing the appropriate concentrations of NaCl into the MRS broth and incubating the cultures at 30 °C for 3 days. Growth in the culture was observed using OD_{600}. L-or D-Lactic acid isomers were identified with the Megazyme assay kit according to the manufacturer's instructions. Lactic acid fermentation type was examined by culturing the strain in MRS broth at 30 °C for 24 h in a Durham tube with the control to collect the gas production. The other biochemical tests were done with API50CHL and APIZYM (bioMérieux) following the manufacturer's instructions. The duplicated test result was read after an incubation time of 48 h.

The fatty acid methyl esters (FAMEs) of S-13^T along with the five reference strains were obtained from cells grown on TSA agar at 30 °C for 2 days. The FAMEs of the strains were extracted from 40 mg of wet cells by four steps: saponification, methylation, extraction, and base washing [14]. The cellular fatty acids were analyzed by gas chromatography using the Microbial Identification System (MIDI 6.0) software package (TSBA6.0).

The cells of S-13^T were Gram-stain-positive, catalase-negative, cocci, facultatively anaerobic and grew well on MRS and TSA agar at 30 °C (Fig. S1, available in the online version of this article). Growth was observed between 15 and 37 °C, but not at 4, 10 and 40 °C. The cells showed a tolerance for 0–3% NaCl and grew at pH 5–7. S-13^T produced L-lactic acid from glucose and utilized D-glucose homofermentatively. The DNA G+C content of S-13^T was 39.4% which was similar to those of the other species in the genus *Lactococcus*.

Phylogenetic analysis of the 16S rRNA of 1379 nt indicated that S-13^T formed a clade with *L. taiwanensis* 0905C15^T and

the four subspecies of *L. lactis*: *L. lactis* subsp. *tructae* L105^T, *L. lactis* subsp. *cremoris* NCDO 607^T *L. lactis* subsp. *hordniae* NBRC 100931^T, and *L. lactis* subsp. *lactis* JCM 5805^T, and the clustering was robust as was evident by the high bootstrap values (Fig. 1). The *rec*A gene of S-13^T showed similarity values of 84.3, 84.0, 83.6, 83.6 and 83.3% to *L. lactis* subsp. *cremoris* NCDO 607^T, *L. lactis* subsp. *hordniae* NBRC 100931^T, *L. lactis* subsp. *lactis* JCM 5805^T, *L. lactis* subsp. *tructae* L105^T and *L. taiwanensis* 0905C15^T, respectively. The maximum-likelihood phylogenetic tree of the *rec*A gene also indicated that S-13^T was distinguishable from the other species in the genus *Lactococcus* (Fig. 2). Comparison of the *rpo*B gene sequences of S-13^T indicated that S-13^T had a sequence similarity of 91.8, 90.6, 90.4, 90.1 and 88.9% to *L. taiwanensis* 0905C15^T, *L. lactis* subsp. *tructae* L105^T, *L. lactis* subsp. *cremoris* NCDO 607^T, *L. lactis* subsp. *hordniae* NBRC 100931^T, and *L. lactis* subsp. *lactis* JCM 5805^T, respectively. S-13^T formed a monophyletic cluster with *L. taiwanensis* NBRC 109049^T and the four subspecies of *Lactococcus lactis*: *L. lactis* subsp. *tructae* L105^T, *L. lactis* subsp. *cremoris* NCDO 607^T, *L. lactis* subsp. *hordniae* NBRC 100931^T, and *L. lactis* subsp. *lactis* JCM 5805^T in the phylogenetic tree for the *rpoB* gene (Fig. 3).

S-13^T shared characteristics that were similar to those of the species in the genus *Lactococcus* such as a facultative anaerobic metabolism, coccus shape, being catalase negative, homofermentative, and utilizing D-glucose to produce L-lactic acid as the final product. However, S-13^T could be separated from the other related species in the genus *Lactococcus* by its specific biochemical and chemotaxonomic characteristics and by the phylogenetic analyses based on the 16S rRNA, *recA* and *rpoB* genes. S-13^T could not grow with a NaCl concentration of 4% or higher, but the other five closely related species could grow with 4% NaCl. The results for carbohydrate fermentation in Table 1 show differences between S-13^T and the other related species. The DNA–DNA relatedness between S-13^T and the closely related type strains were 25.6, 20.4, 25.1, 20.2 and 21.7% to *L. taiwanensis* 0905C15^T, *L. lactis* subsp. *tructae* L105^T, *L. lactis* subsp. *cremoris* NCDO 607^T, *L. lactis* subsp. *hordniae* NBRC 100931^T, and *L. lactis* subsp. *lactis* JCM 5805^T, respectively, which were lower than the threshold for the differentiation of a novel species [15]. Digital DNA–DNA hybridization (dDDH) was also tested using the Genome Blast Distance Phylogeny version 2.1 web browser from DSMZ (http://ggdc.dsmz.de). The dDDH relatedness between S-13^T and the related type species were 22.4, 22.5, 22.8 and 23.2% for *L. lactis* subsp. *tructae* DSM 21502^T (NZ_JXKC00000000), *L. lactis* subsp. *hordniae* DSM 20450^T(NZ_JXKA00000000), *L. lactis* subsp. *cremoris* NBRC 100676^T (NZ_BCVK00000000) and *L. lactis* subsp. *lactis* NBRC 100933^T (NZ_BCNL00000000), respectively [16].

Moreover, the cellular fatty acid profile could be used to distinguish S-13^T from the other members of the genus *Lactococcus*. The predominant cellular fatty acids (>10%) of S-13^T were $C_{16:0}$ (46.4%), $C_{19:0}$ cyclo-ω8*c* (23.6%), and $C_{14:0}$ (15.3%); the minor fatty acids were cyclo-$C_{17:0}$ (1.6%), $C_{18:0}$ (0.7%) and summed featured 8 (6.8%), and 3 (4.9%). However, compared with the other five reference species, the levels of $C_{16:0}$ and summed feature 3 were much higher than those in *L. taiwanensis* 0905C15^T, *L. lactis* subsp. *tructae* L105^T, *L.lactis* subsp. *cremoris* NCDO 607^T, *L. lactis* subsp. *hordniae* NBRC 100931^T, and *L. lactis* subsp. *lactis* JCM 5805^T. Significant differences in the fatty acid profiles were found between the isolate and the reference strains and are shown in Table 2.

Table 2. The cellular fatty acids of S-13^T and closely related species of the genus *Lactococcus*

Strain:S-13^T; 2, *Lactococcus taiwanensis* 0905C15^T; 3, *Lactococcus lactis* subsp. *tructae* L105^T; 4, *Lactococcus lactis* subsp. *cremoris* NCDO 607^T; 5, *Lactococcus lactis* subsp. *hordniae* NBRC 100931^T; 6, *Lactococcus lactis* subsp. *lactis* JCM 5805^T. Fatty acids that represented <0.5% of the total are not included in this table. Summed feature 3 contains $C_{16:1}\omega7c$/ $C_{16:1}\omega6c$; summed feature 8 contains $C_{18:1}\omega7c$/ $C_{18:1}\omega6c$.

Fatty acids	1	2	3	4	5	6
Saturated						
$C_{14:0}$	15.3	8.7	21.3	8.6	5.0	8.7
$C_{16:0}$	46.4	45.0	40.1	42.0	39.9	27.2
$C_{18:0}$	0.7	3.1	0.5	1.7	2.2	0.6
Unsaturated						
cyclo-$C_{17:0}$	1.6	1.8	1.4	1.1	—	1.3
cyclo-$C_{19:0}\omega8c$	23.6	33.0	24.0	32.8	—	43.7
Summed feature						
3	4.9	3.0	3.5	2.4	2.4	4.3
8	6.8	5.3	8.9	9.9	48.3	12.3

In conclusion, based on the results of the phylogenetic analyses and phenotypic, biochemical, and chemotaxonomic characteristics, strain S-13^T represents a novel species of the genus *Lactococcus*, and the name *Lactococcus kimchii* sp. nov. is proposed. The type strain is S-13^T (=KCTC 21096^T=NBRC 113348^T) isolated from kimchi in Korea.

DESCRIPTION OF *LACTOCOCCUS KIMCHII* SP. NOV.

Lactococcus kimchii (kim′chi.i. N.L. gen. n. *kimchii* of kimchi, a Korean fermented vegetable).

Cells are Gram-stain-positive, catalase negative, cocci-shaped, facultatively anaerobic and grow well anaerobically on MRS and TSA agar at 30 °C. Cells utilize D-glucose homofermentatively but do not produce gas. L-lactic acid is the main product. Growth occurs at 15–37 °C but not at 10 or 45 °C. The cells can grow at pH 5–7 and have a tolerance for NaCl from 0 to 3%. Optimum growth conditions are 25–30 °C, pH 6–7 and 0–1 % NaCl. The enzyme reaction is positive for acid phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), α-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphate-hydrolase, and valine arylamidase and weakly positive for lipase (C14) but is negative for N-acetyl-β-glucosaminidase, alkaline phosphatase, α-chymotrypsin,

α-fucosidase, α-galactosidase, α-mannosidase, β-glucosidase, β-glucuronidase, and trypsin. Acid is produced from amygdalin, L-arabinose, arbutin, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, salicin, cellobiose, maltose, lactose, melibiose, trehalose, starch, gentiobiose, and D-xylose but is not produced from D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, gluconate, glycerol, glycogen, L-fucose, D-fucose, inositol, inulin, D-lyxose, melezitose, L-rhamnose, L-sorbose, D-sorbitol, sucrose, D-tagatose, turanose, L-xylose, and xylitol. The major fatty acids are $C_{16:0}$, cyclo-$C_{19:0}\omega 8c$, $C_{14:0}$, and summed features 8 and 3.

The type strain is S-13^T (=NBRC 113348^T=KCTC 21096^T), isolated from kimchi in Korea. The DNA G+C content of the type strain is 39.4 mol%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain is MG576055 and the numbers for the whole genome sequence SDAK01000001, SDAK01000002, SDAK01000003 and SDAK01000004, respectively.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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