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Lactobacillus kimchii sp. nov., a new species from kimchi

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A bacteriocin-producing lactic acid bacterium, which was isolated from the Korean fermented-vegetable food kimchi, was subjected to a polyphasic taxonomic study using phenotypic characterization and phylogenetic and genetic methods. This organism (MT-1077^T) has phenotypic properties that are consistent with the description characterizing the genus *Lactobacillus*. Phylogenetic analysis based on 16S rDNA sequences showed clearly that strain MT-1077^T is a member of the genus *Lactobacillus*. The closest phylogenetic relatives are *Lactobacillus alimentarius* KCTC 3593^T and *Lactobacillus farciminis* LMG 9200^T, with levels of 16S rDNA similarity of 98·4 and 98·2%, respectively. Levels of 16S rDNA similarity between strain MT-1077^T and other *Lactobacillus* species were less than 93·0%. Differences in some phenotypic characteristics and DNA–DNA relatedness data indicated that strain MT-1077^T should be distinguished from *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T. On the basis of the data presented, it is proposed that strain MT-1077^T should be placed in the genus *Lactobacillus* as a new species, *Lactobacillus kimchii* sp. nov. The type strain of the new species is strain MT-1077^T (= KCTC 8903P^T = JCM 10707^T).

Keywords: *Lactobacillus kimchii* sp. nov., kimchi, lactic acid bacterium

INTRODUCTION

Kimchi is one of the representative foods of Korea. It is prepared with various kinds of vegetables, spices and other ingredients and becomes palatable through proper fermentation. Kimchi fermentation is initiated by various micro-organisms present in the raw materials, but the fermentation is gradually dominated by lactic acid bacteria (Cheigh & Park, 1994; Lee, 1991). Lactic acid bacteria play an important role in the taste of kimchi, as in dairy products such as cheese and fermented milk. It has been reported that strains belonging to the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Lactococcus* exist in kimchi (Cheigh & Park, 1994; Mheen & Kwon, 1984; So & Kim, 1995; Lee *et al.*, 1997). Accordingly, kimchi has been one of the important sources of lactic acid bacteria in Korea for a long time. Generally, lactic acid bacteria are known to have the potential to inhibit the growth of micro-organisms, especially pathogenic and spoilage bacteria. The antimicrobial activity of lactic acid bacteria is known to be due to organic acids, hydrogen peroxide, diacetyl and bacteriocins (Dahiya & Speck, 1968; Jay, 1982; Klaenhammer, 1988). Many lactic acid bacteria have been isolated from kimchi in Korea and some of them have been shown to have antimicrobial activity and other useful properties (Choi & Beuchat, 1994; Kim & Park, 1995; Lee *et al.*, 1999). Nevertheless, systematic studies have rarely been performed for the reliable classification and identification of these strains (Lee *et al.*, 1997). It is generally recognized that phylogenetic analysis based on 16S DNA sequence and genetic relatedness, together with extensive phenotypic characteristics, are also very important for reliable classification and identification of lactic acid bacteria (Vandamme *et al.*, 1996).

Recently, a novel bacteriocin-producing lactic acid bacterium (strain MT-1077^T) was isolated from a kind

The GenBank accession number for the 16S rDNA sequence of strain MT-1077^T is AF183558.

of kimchi. In this work, we describe the phenotypic, phylogenetic and genetic characteristics of the isolate. On the basis of data described below, a new species name for strain MT-1077^T, *Lactobacillus kimchii* sp. nov., is proposed.

METHODS

Bacterial strains and culture conditions. Various types of kimchi were used as sources for the isolation of lactic acid bacteria. Strain MT-1077^T was isolated from homogenized kimchi spread onto MRS medium (Difco). Strain MT-1077^T and two reference strains, *Lactobacillus alimentarius* KCTC 3593^T and *Lactobacillus farciminis* LMG 9200^T, were mainly cultivated aerobically at 30 °C on MRS medium for the investigation of morphological and physiological characteristics. The cell masses for DNA extraction were produced from liquid MRS medium. All strains were cultivated aerobically at 30 °C on a horizontal shaker at 150 r.p.m. Strain MT-1077^T and the two reference strains were also cultivated at 30 °C for 3 d on MRS agar for fatty acid methyl ester analysis.

Phenotypic characterization. The morphology of cells was examined by phase-contrast microscopy and transmission electron microscopy. Catalase activity was determined by using 3 % (v/v) hydrogen peroxide. Hydrolysis of casein and

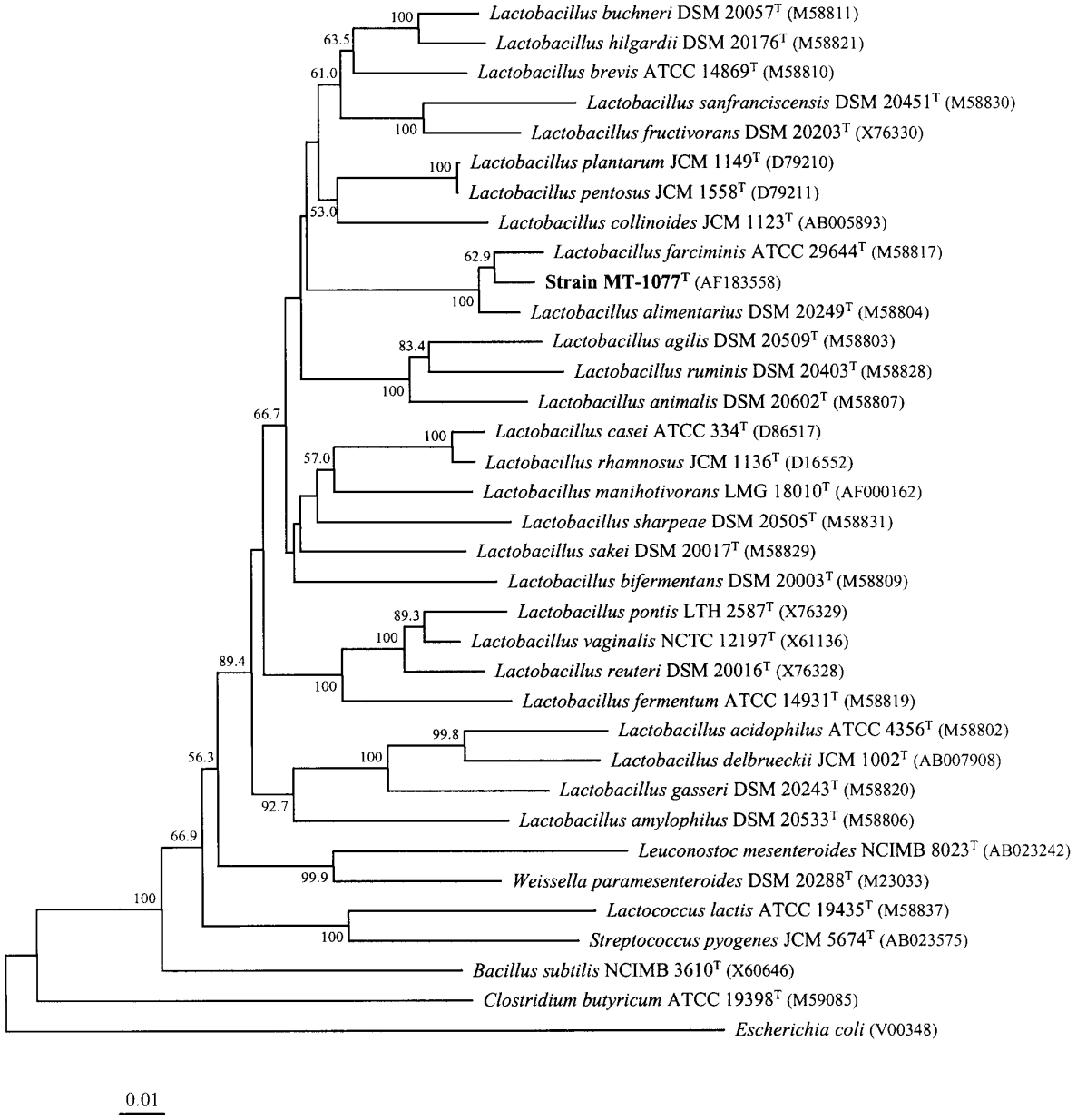

Fig. 1. Phylogenetic tree showing the positions of strain MT-1077^T, some *Lactobacillus* species and representative strains of other related genera based on 16S rDNA sequences. Scale bar represents 0·01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replicates) greater than 50 % are shown at the branch points.

starch was determined according to Cowan & Steel (1965) using solid MRS medium. The configuration and concentration of lactic acid were determined by using the D/L-lactate enzymic kit (Boehringer Mannheim). Gas production from glucose was examined with Durham tubes. Tolerance of NaCl and growth at various temperatures were tested on MRS medium. Sugar fermentation patterns were determined by using the API 50 CHL system (bioMérieux). Other physiological tests were performed with the API 20NE system (bioMérieux). Fatty acids were extracted and analysed according to the instructions of the Microbial Identification System (MIDI).

Isolation of DNA. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al*., 1996), with the exception that ribonuclease T1 was used together with ribonuclease A.

Determination of G+C content. The DNA G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC.

DNA–DNA relatedness test. DNA–DNA hybridization studies were performed by using the fluorometric method described by Ezaki *et al*. (1989), using photobiotin-labelled DNA probes and microdilution wells. The following procedure was used: 25 µl purified DNA sample (200 µg ml^{-1}) was denatured by boiling for 5 min and then diluted to 10 µg ml^{-1} with cold PBS containing 0·1 M $MgCl_2$. The diluted DNA suspension was distributed into five wells of a microplate (Nunc) at 100 µl per well and the plate was incubated for 8 h at 30 °C. Express DNA suspensions were discarded and the plate was dried for 30 min at 45 °C. The plate was prehybridized for 10 min followed by hybridization with biotinylated DNA at 45 °C for approximately 10 h. The plate was washed three times with 1× SSC and 100 µl streptavidin-D-galactosidase (Gibco-BRL) (diluted 1000-fold with PBS plus 0·5 % BSA) was added to each well. The plate was incubated at 37 °C for 30 min and washed three times with 1× SSC. Finally, 100 µl 4-methylumbellifery1 β-D-galactopyranoside (10 mg substrate ml^{-1} dimethylformamide diluted 100-fold with PBS) was added to the wells and the plate was incubated at 37 °C for 30 min. The fluorescence intensity was read with a Labsystems fluoroskan II at wavelengths of 360 nm for excitation and 450 nm for emission. The highest and lowest values in each sample were excluded and the remaining three values were used for the calculation of similarity values. DNA relatedness values are the mean of three values.

16S rDNA sequencing and phylogenetic analysis. The 16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al*., 1998). The PCR product was purified by using a QIAquick PCR purification kit (Qiagen). The purified 16S rDNA was sequenced using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically on an Applied Biosystems model 310 automatic DNA sequencer. The 16S rDNA sequence of strain MT-1077^T was aligned with 16S rRNA gene sequences of *Lactobacillus* species and the representatives of some related genera by using the CLUSTAL W software (Thompson *et al*., 1994). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analyses. Evolutionary distance matrices were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) as implemented within the NEIGHBOR program of the same package. The stability of relationships was assessed by bootstrap analysis of 1000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

Nucleotide sequence accession numbers. GenBank and EMBL accession numbers for reference 16S rRNA gene sequences used in the phylogenetic analysis are represented in Fig. 1.

RESULTS

Morphological characteristics

Strain MT-1077^T is Gram-positive, non-spore-forming and non-motile. The cells are short, slender rods measuring 0·6–0·8 × 1·5–3·0 µm in MRS medium at 30 °C and occur singly, in pairs or occasionally in short chains. After incubation on MRS agar for 3 d, colonies are white, circular to slightly irregular, convex, smooth, opaque and approximately 0·8–1·5 mm in diameter.

Physiological and biochemical characteristics

Strain MT-1077^T exhibited no oxidase or catalase activities. Casein and aesculin were hydrolysed but gelatin, starch and urea were not. Nitrate was not reduced to nitrite. Arginine was not deaminated and indole was not produced. Strain MT-1077^T produced L(+)-lactic acid and D(−)-lactic acid. Strain MT-1077^T fermented gluconate and did not produce gas from glucose, indicating that this organism is facultatively heterofermentative (Vandamme *et al*., 1996). Growth occurred well in aerobic and strict anaerobic conditions on liquid and solid MRS media. Strain MT-1077^T grew at 10 and 40 °C but not at 45 °C. The optimal temperature for growth was approximately 30 °C. Strain MT-1077^T grew optimally at pH 6·0–7·0 and growth was inhibited at pH 4·0 and 9·0. Strain MT-1077^T grew in the presence of 8 % (w/v) NaCl but not in the presence of 10 % (w/v) NaCl. Some other properties of strain MT-1077^T are summarized in Table 1, together with those of *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T.

Fatty acid analysis

Strain MT-1077^T showed a cellular fatty acid profile similar to those reported for *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T (Table 2). The major fatty acid of strain MT-1077^T was an unsaturated fatty acid, $C_{18:1}$ ω9c (50·5 %), and a significant amount of $C_{16:0}$ (11·6 %) was also detected.

DNA base composition

The G+C contents of strain MT-1077^T, *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T were determined by the nuclease P1 and HPLC method in this study. The G+C content of strain MT-1077^T

Table 1. Physiological characteristics of strain MT-1077^T, *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T

+, Positive reaction; −, negative reaction; w, weakly positive reaction. All strains were positive for the following characters: hydrolysis of aesculin and casein; fermentation of glucose, fructose, mannose, *N*-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, maltose, sucrose and trehalose; growth on MRS medium at 30, 37 and 40 °C; and growth in the presence of 2, 4, 6 and 8 % NaCl. All strains were negative for the following characters: catalase activity; hydrolysis of gelatin, starch and urea; production of indole; reduction of nitrate to nitrite; gas production from glucose; fermentation of glycerol, erythritol, D-arabinose, L-xylose, adonitol, *β*-methyl D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl D-mannoside, melibiose, inulin, raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate and 5-ketogluconate; growth on MRS medium at 45 °C. G+C contents were determined by HPLC.

Characteristic	Strain MT-1077^T	*L. alimentarius* KCTC 3593^T	*L. farciminis* LMG 9200^T
NH_3 production from arginine*	−	−	+
Lactic acid isomers†	DL	L(D)	L(D)
Fermentation of:			
L-Arabinose	+	+	−
Ribose	+	+	−
D-Xylose	+	−	−
Galactose	w	+	+
α-Methyl D-glucoside	−	−	+
Amygdalin	+	+	w
Lactose	−	−	+
Melezitose	+	−	−
Gentiobiose	+	+	−
D-Turanose	−	−	+
D-Tagatose	−	−	+
Gluconate	+	+	−
Growth on MRS medium at:			
10 °C	+	w	−
15 °C	+	+	w
Growth in the presence of 10 % NaCl	−	−	+
DNA G+C content (mol%)	35	35	36

* Data for *L. alimentarius* and *L. farciminis* are from Kandler & Weiss (1986).

† DL, 25–75 % of total lactic acid is of the L configuration; L (D), the D isomer makes up 15–20 % of the total lactic acid (Kandler & Weiss, 1986). Data for *L. alimentarius* and *L. farciminis* are from Kandler & Weiss (1986).

was 35 mol % and the G+C contents of *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T were 35 and 36 mol %, respectively.

Phylogenetic analysis

The 16S rDNA of strain MT-1077^T was sequenced directly following PCR amplification. This almost complete 16S rDNA sequence determined was 1522 bp long, which corresponded to the region between positions 28 and 1524 of the 16S rDNA of *Escherichia coli*. This sequence was subjected to similarity searches with public databases to infer a possible phylogenetic classification of strain MT-1077^T. The result revealed that strain MT-1077^T is a member of the genus *Lactobacillus*. This became clear from the phylogenetic analysis and nucleotide sequence similarity values. The phylogenetic tree showed that strain MT-1077^T forms an evolutionary lineage within the radiation of a cluster comprising *Lactobacillus* species and is phylogenetically most closely related to *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T (Fig. 1). Levels of 16S rDNA similarity between strain MT-1077^T and *L. alimentarius* KCTC 3593^T and between strain MT-1077^T and *L. farciminis* LMG 9200^T were 98·4 and 98·2 %, respectively. The cluster of strain MT-1077^T, *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T was differentiated from phylogenetic lineages comprising other *Lactobacillus* species, although it was not supported by a high bootstrap resampling value (Fig. 1). However, the relationship between the cluster of strain MT-1077^T, *L. farciminis* LMG 9200^T and *L. alimentarius* KCTC 3593^T was supported by a bootstrap resampling value of 100 %. Strain MT-1077^T exhibited levels of 16S rDNA similarity below 93·0 % to other *Lactobacillus* species the 16S rDNA sequences of which are known. Levels of 16S rDNA similarity between strain MT-1077^T and representatives of other related genera were as follows: *Leuconostoc mesenteroides* NCDO 523^T (85·1 %),

Table 2. Cellular fatty acid profiles of strain MT-1077^T, *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T

Percentages of total fatty acids are shown. c, *cis*; t, *trans*.

Fatty acid	Strain MT-1077^T	*L. almentarius* KCTC 3593^T	*L. farciminis* LMG 9200^T
Saturated fatty acids:			
$C_{12:0}$	—	—	0·4
$C_{14:0}$	3·8	4·2	3·1
$C_{16:0}$	11·6	22·8	13·0
$C_{18:0}$	2·0	—	1·8
Unsaturated fatty acids:			
$C_{17:1}$ ω8c	—	—	1·3
$C_{18:1}$ ω9c	50·5	36·1	43·3
Branched-chain fatty acid:			
iso-$C_{19:0}$	3·4	—	—
Summed features:*			
1	—	—	0·5
4	6·9	6·9	6·1
6	—	—	1·1
7	8·6	6·2	8·4
9	13·3	23·9	21·1

*Summed features represent groups of fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 contained one or more of the following fatty acids: $C_{14:1}$ ω5t and/or $C_{14:1}$ ω5c. Summed feature 4: $C_{16:1}$ ω7c and/or *iso*- $C_{15:0}$ 2OH. Summed feature 6: $C_{18:2}$ ω6,9c and/or *anteiso*-$C_{18:0}$. Summed feature 7: $C_{18:1}$ ω7c, $C_{18:1}$ ω9t and/or $C_{18:1}$ ω12t. Summed feature 9: unknown 18·846, unknown 18·858 and/or $C_{19:0}$ cyclo.

Table 3. Levels of DNA–DNA relatedness between strain MT-1077^T, *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T

Percentage reassociation is shown.

Taxon	Strain MT-1077^T	*L. alimentarius* KCTC 3593^T	*L. farciminis* LMG 9200^T
Strain MT-1077^T	100	10·9	8·1
L. alimentarius KCTC 3593^T	6·3	100	5·3
L. farciminis LMG 9200^T	7·1	7·4	100

Weissella paramesenteroides DSM 20288^T (87·9 %), *Lactococcus lactis* ATCC 19435^T (85·9 %) and *Streptococcus pyogenes* JCM 5674^T (86·6 %).

DNA–DNA relatedness

DNA–DNA relatedness tests were performed between strain MT-1077^T, *Lactobacillus alimentarius* KCTC 3593^T and *Lactobacillus farciminis* LMG 9200^T. Strain MT-1077^T and *L. alimentarius* KCTC 3593^T exhibited two independent levels of DNA–DNA relatedness of 6·3 and 10·9 % (Table 3). Two independent determinations between strain MT-1077^T and *L. farciminis* LMG 9200^T yielded values of 7·1 and 8·1 % (Table 3).

DISCUSSION

Lactic acid bacteria have been isolated from a variety of habitats such as foods, vegetation, sewage, humans and animals (Kandler & Weiss, 1986; Hammes *et al.*, 1992). It has also been reported that numerous lactic acid bacteria exist dominantly in the Korean fermented-vegetable food kimchi (Cheigh & Park, 1994; So & Kim, 1995; Lee *et al.*, 1997). Nevertheless, few extensive taxonomic studies have been conducted on the microbial flora of kimchi. Recent preliminary studies have suggested the existence of taxonomically interesting strains in kimchi. Strain MT-1077^T is one of such strains and, moreover, was shown to produce a novel bacteriocin. Accordingly, this strain was subjected to the present study to investigate the possibility of the first report of a new taxon in kimchi. The morphological and physiological characteristics obtained for strain MT-1077^T are in accordance with the description of the genus *Lactobacillus*. Lactobacilli are characterized as Gram-positive, facultatively anaerobic, catalase-negative, non-spore-forming rods that produce lactic acid as the major fermentation product (Hammes *et al.*, 1992). Lactobacilli have a DNA G+C content of 32–55 mol % and do not usually reduce nitrate (Hammes *et al.*, 1992). Accordingly, it is suggested by phenotypic character-

ization that strain MT-1077^T appears to be a member of the genus *Lactobacillus*. Nevertheless, phylogenetic analysis based on 16S rDNA sequences should be performed to verify this suggestion, since it is currently one of the most useful methods for estimating the relationships between genera belonging to the lactic acid bacteria and for assigning an isolate to such genera (Collins *et al*., 1991; Vandamme *et al*., 1996). The result of phylogenetic inference was in good agreement with the results obtained from phenotypic characterization. A phylogenetic tree based on 16S rDNA sequences shows that strain MT-1077^T falls within the radiation of a cluster comprising *Lactobacillus* species (Fig. 1).

Strain MT-1077^T exhibited the closest phylogenetic affinity to *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T, with levels of 16S rDNA similarity of 98·4 and 98·2 %, respectively. However, levels of 16S rDNA similarity between strain MT-1077^T and other *Lactobacillus* species were low (below 93 %), indicating that strain MT-1077^T is a different species from these *Lactobacillus* species according to the available compilation of data by Stackebrandt & Goebel (1994). Accordingly, comparative taxonomic studies were performed among strain MT-1077^T, *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T to determine whether strain MT-1077^T could be considered as a new species of the genus *Lactobacillus* or would be assigned to one of the two species. Strain MT-1077^T showed differences from the two species in some physiological characteristics such as fermentation of sugars (Table 1). Strain MT-1077^T was differentiated metabolically from *L. farciminis* LMG 9200^T. Strain MT-1077^T was considered to be facultatively heterofermentative, like *L. alimentarius* KCTC 3593^T (Vandamme *et al*., 1996; Kandler & Weiss, 1986), whereas *L. farciminis* LMG 9200^T is obligately homofermentative (Kandler & Weiss, 1986). The fatty acid profile of strain MT-1077^T was similar to those of *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T, but showed slight differences from those of the two *Lactobacillus* species in the amounts of some fatty acids (Table 2). Levels of DNA–DNA relatedness show clearly that strain MT-1077^T differs from *L. alimentarius* KCTC 3593^T and *L. farciminis* LMG 9200^T (Wayne *et al*., 1987). Based on the differences in some phenotypic characteristics, phylogenetic inference and genetic distinctiveness, strain MT-1077^T should be placed as a new species of the genus *Lactobacillus*. Therefore, we propose a new species name, *Lactobacillus kimchii* sp. nov., for strain MT-1077^T.

Description of *Lactobacillus kimchii* sp. nov.

Lactobacillus kimchii (kim′chi.i. M.L. n. *kimchii* from kimchi, a Korean fermented-vegetable food).

Cells are short, slender rods measuring 0·6–0·8 × 1·5–3·0 μm in MRS medium at 30 °C, which occur singly, in pairs or occasionally in short chains. Gram-positive, non-spore-forming and non-motile. After 3 d incubation on MRS agar, colonies are white, circular to slightly irregular, convex, smooth, opaque and approximately 0·8–1·5 mm in diameter. Catalase-negative. Casein and aesculin are hydrolysed but gelatin, starch and urea are not. Nitrate is not reduced to nitrite. Arginine is not deaminated. Indole is not produced. L(+)-Lactic acid and D(−)-lactic acid are produced. Facultatively heterofermentative. Facultatively anaerobic. Growth occurs at 10 and 40 °C but not at 45 °C. Optimal temperature and pH for growth are approximately 30 °C and 6·0–7·0. Grows in the presence of 8 % NaCl but not in the presence of 10 % NaCl. Acid is produced from *N*-acetylglucosamine, aesculin, L-arabinose, amygdalin, arbutin, cellobiose, fructose, galactose, gentiobiose, gluconate, glucose, maltose, mannose, melezitose, ribose, salicin, sucrose, trehalose and D-xylose. The major fatty acid is the unsaturated fatty acid $C_{18:1}$ ω9c. The G+C content is 35 mol % (determined by HPLC). Isolated from kimchi, a Korean fermented-vegetable food. The type strain is strain MT-1077^T, which has been deposited in the Korean Collection for Type Cultures as KCTC 8903P^T and Japan Collection of Microorganisms as JCM 10707^T.

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