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# *Leuconostoc kimchii* sp. nov., a new species from kimchi

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**A Gram-positive, catalase-negative, facultatively anaerobic, coccus-shaped bacterium, designated IH25$^T$, was isolated from kimchi, a traditional Korean vegetable product. Phylogenetic analysis based on almost complete 16S rDNA sequences placed the isolate in a monophyletic clade corresponding to the genus *Leuconostoc*. All validly described species in the genus *Leuconostoc*, with the exception of *Leuconostoc fallax*, showed high sequence identity of over 97%. The 16S rDNA sequence of strain IH25$^T$ showed the highest homology to those of *Leuconostoc gelidum* DSM 5578$^T$ (98·9%) and *Leuconostoc citreum* KCTC 3526$^T$ (98·3%). However, DNA–DNA hybridization experiments indicated that the organism represents a novel genomic species in the genus, since the previously known leuconostocs share DNA homology with strain IH25$^T$ of less than 70%. In this work, it is proposed that isolate IH25$^T$ be classified in the genus *Leuconostoc* as *Leuconostoc kimchii* sp. nov. The type strain of *Leuconostoc kimchii* is IH25$^T$ (= KCTC 2386$^T$ = IMSNU 11154$^T$).**

**Keywords:** *Leuconostoc kimchii* sp. nov., kimchi, taxonomy, lactic acid bacteria

## INTRODUCTION

The genus *Leuconostoc* encompasses a phylogenetically coherent group of lactic acid bacteria and currently consists of eight species, namely *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Leuconostoc gelidum*, *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc pseudomesenteroides*, *Leuconostoc fallax* and *Leuconostoc argentinum* (Holzapfel & Schillinger, 1992; Dicks *et al*., 1993, 1995). *L. mesenteroides* contains three subspecies, *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum* and *L. mesenteroides* subsp. *cremoris* (Garvie, 1983). Leuconostocs are Gram-positive, facultatively anaerobic, asporogenous, catalase-negative, spherical organisms containing DNA with relatively low G+C content (37–45 mol%), which produce lactic acid as a main end-product of fermentation and in many cases produce dextran. They thrive in a variety of environments including fermented foods, such as dairy and meat products (Garvie, 1986; Holzapfel & Schillinger, 1992). Some species have been reported to play an important role in the fermentation of plant materials. For example, *L. mesenteroides* subsp. *mesenteroides* initiates the fermentation of sauerkraut and a number of traditional fermented foods in tropical regions (Stamer, 1975; Puspito & Fleet, 1985; Gashe, 1987).

Kimchi, a traditional Korean food, is a well-known lactic-acid-fermented vegetable product, made of Chinese cabbage, radishes and cucumbers. A typical Korean adult consumes an average of 50–200 g of kimchi per day. Taxonomically diverse groups of lactic acid bacteria have been found in the fermentation process of kimchi. Some important species responsible for the fermentation of kimchi are leuconostocs such as *L. mesenteroides*, *L. pseudomesenteroides* and *L. lactis*, as well as lactobacilli including *Lactobacillus brevis* and *Lactobacillus plantarum* (Lee *et al*., 1993, 1997). Most taxonomic studies on bacterial isolates from kimchi have been based on limited phenotypic methods, especially biochemical properties such as sugar fermentation patterns. It is clear that a polyphasic approach is needed to pinpoint the accurate taxonomic position of isolates that may play an important role in the fermentation process in kimchi.

In recent work, several *Leuconostoc*-like bacteria were isolated and identified using a battery of biochemical methods. Among them, a strain was found to have a

The GenBank accession numbers for the 16S rDNA sequences of strain IH25$^T$, *Leuconostoc citreum* KCTC 3526$^T$, *Leuconostoc argentinum* DSM 8581$^T$ and *Leuconostoc gelidum* DSM 5578$^T$ are AF173986, AF111949, AF175403 and AF175402, respectively.

unique biochemical pattern which did not fit the description of any valid species in the genus *Leuconostoc*. This strain was therefore subjected to further taxonomic study. In this paper, we present the polyphasic characteristics of this isolate, which we name *Leuconostoc kimchii* sp. nov.

## METHODS

**Bacterial strains.** A kimchi sample, made from Chinese cabbage, was collected at the initial stage of fermentation, i.e. the first 5 days, at 20 °C. Bacterial strains were isolated using sucrose-agar plates at 20 °C (Garvie, 1984). The resultant pure cultures were grown in MRS broth (Difco) at 30 °C for 24 h and stored in 10 % (v/v) dimethyl sulfoxide at −70 °C. An isolate, designated IH25$^T$, was shown to have a distinctive biochemical pattern and was chosen for further studies. The type strains used in this study are *L. argentinum* DSM 8581$^T$, *L. carnosum* DSM 5576$^T$, *L. citreum* KCTC 3526$^T$, *L. fallax* DSM 20189$^T$, *L. gelidum* DSM 5578$^T$, *L. mesenteroides* subsp. *mesenteroides* KCTC 3505$^T$, *L. lactis* KCTC 3528$^T$ and *L. pseudomesenteroides* DSM 20193$^T$. All strains except *L. gelidum* were cultivated at 30 °C, unless otherwise stated.

**Biochemical and physiological tests.** The strains were characterized biochemically using the API CH50 strip and API CHL medium systems according to the manufacturer's instructions (API bioMérieux). All test preparations were incubated for 48 h before reading colour changes. Determination of the optical isomer of lactic acid by using lactate dehydrogenases was carried out according to the manufacturer's instructions (TC D-/L-lactic acid, Boehringer Mannheim) (Okada *et al.*, 1978). Arginine dihydrolase activity was tested by using 1 % L-arginine monohydrochloride (Smibert & Krieg, 1994).

**Cellular fatty acid analysis.** Fatty acid methyl esters were prepared from biomass that was scraped from MRS agar incubated for 24 h at 30 °C. The composition of whole-cell fatty acids was determined using the MIDI system (Hewlett Packard; Sasser, 1990).

**16S rDNA sequencing.** Chromosomal DNA was isolated by a modification of the method of Varmanen *et al.* (1998). Mid-exponential-phase cells ($OD_{600} = 0{\cdot}4$) in 3 ml MRS broth containing 1 % (w/v) glycine were pelleted and resuspended in 380 µl 6·7 % sucrose solution (in TE buffer; 50 mM Tris/HCl, 1 mM EDTA, pH 8·0). One hundred microlitres lysozyme (50 mg ml$^{-1}$) and 5 µl RNase A (10 mg ml$^{-1}$) were added and the cells were incubated at 37 °C for 1 h. Cells were lysed by adding 30 µl 20 % SDS (in TE buffer). Cell lysates were incubated by adding 20 µl proteinase K (17 mg ml$^{-1}$) at 50 °C for 1 h. Cell lysates were diluted fourfold with buffer (10 mM Tris/HCl pH 8·0, 1 mM EDTA) and were then subjected to phenol extraction and ethanol precipitation. PCR amplification of the 16S rDNA was performed in a 100 µl reaction containing 2·5 U *Taq* polymerase by using the following program: 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min and an additional extension step at 72 °C for 10 min. The primers used were 5′-GAGTTTGATCCTGGCTCAG-3′ (*Escherichia coli* numbering system, positions 9–27) and 5′-AGAAAGGAGGTGATCCAGCC-3′ (positions 1525–1544) (Brosius *et al.*, 1978; Suzuki & Yamasato, 1994). PCR products were cloned into pGEM vector (Promega) and sequenced by using a dideoxy sequencing kit (Sequenase version 2·0, USB).

**Phylogenetic analysis.** The resultant 16S rDNA sequence of strain IH25$^T$ was aligned manually against representative sequences of lactic acid bacteria obtained from GenBank using information on secondary structure. Unrooted evolutionary trees were inferred by using three treeing algorithms, namely, the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. The distance model of Jukes & Cantor (1969) was used to generate an evolutionary distance matrix. The topologies of the resulting trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. All phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 1993).

**DNA–DNA relatedness studies.** DNA relatedness among strains was determined in duplicate experiments by using the membrane filter technique modified after Chun *et al.* (1998). Genomic DNA (200 ng) was denatured by using the alkaline method and immobilized on nylon membrane (Hybond-N$^+$; Amersham) by applying a low vacuum. DNA preparations were labelled with the ECL direct nucleic acid labelling and detection kit (RPN 3000; Amersham). One hundred nanograms of the denatured DNA samples was labelled with glutaraldehyde according to the manufacturer's protocol. The membranes were prehybridized in hybridization solution at 42 °C for 2 h. Hybridization was carried out in 10 ml hybridization solution containing labelled DNA (10 ng ml$^{-1}$) at 42 °C for 2 h. After hybridization, the nylon filters were washed twice in primary wash solution (0·4 % SDS, 0·1 × SSC) at 42 °C and subsequently washed twice with secondary wash solution (2 × SSC). Detection reagents were added to the membrane blots for 1 min at room temperature and were then drained off. Membrane blots wrapped in SaranWrap were exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 0·5–2 min. The signal intensities were determined by using an image analyser (Pharmacia). The signal produced by self-hybridization was taken as 100 % and percentage homology values were calculated for the duplicate samples.

**Determination of DNA base composition.** DNA was prepared according to Chun & Goodfellow (1995). The G+C contents of the resultant preparations were determined by the thermal denaturation method (Mandel & Marmur, 1968).

## RESULTS AND DISCUSSION

Isolate IH25$^T$ showed phenotypic characteristics typical of leuconostocs, i.e. a Gram-positive, non-spore-forming, coccus-shaped, facultatively anaerobic, catalase-negative organism that usually occurs as single cells or in pairs. The G+C content of the organism, 37 mol %, was within the observed range for the genus *Leuconostoc*, i.e. 37–45 mol % (Garvie, 1983). As is typical of leuconostocs, more than 95 % of the lactate produced by strain IH25$^T$ was the D-(−) isomer and the strain did not hydrolyse arginine. The organism contained major amounts of straight-chain saturated, monounsaturated and cyclopropane-ring acids: $C_{16:0}$ (35 %), $C_{19\text{-}cyclo}$ (29 %), $C_{18:1}$ (16 %), $C_{16:1}$ (10 %) and $C_{14:0}$ (7 %). The presence of large amounts of the $C_{19}$ cyclopropane-ring acid and its precursor ($C_{18:1}$) was also reported in other leuconostocs (Shaw & Harding, 1989). Our isolate has a profile most similar to those of *L. gelidum* and *L. carnosum*. However, strain IH25$^T$

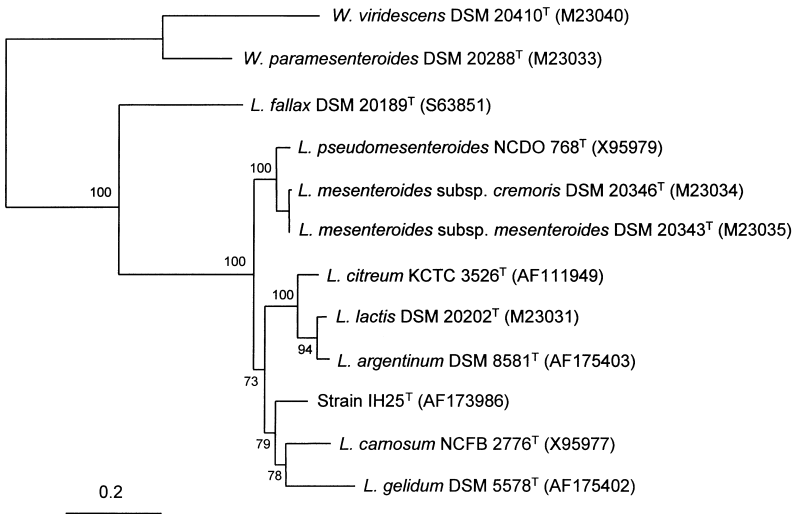


***Fig. 1.*** Unrooted neighbour-joining tree based on nearly complete 16S rDNA sequences showing relationships between strain IH25$^T$ and members of the genus *Leuconostoc*. Numbers at the nodes indicate levels of bootstrap support based on neighbour-joining analyses of 1000 resampled datasets. The scale bar indicates 0.2 nucleotide substitutions per nucleotide position. *W.*, *Weissella*.

***Table 1.*** Mean levels of DNA relatedness (%) of strain IH25$^T$ to *Leuconostoc* strains

| Strain | Labelled strain | | |
|---|---|---|---|
| | **IH25$^T$** | **DSM 5578$^T$** | **DSM 20193$^T$** |
| Strain IH25$^T$ | 100 | 40 | 2 |
| *L. argentinum* DSM 8581$^T$ | 7 | 10 | 4 |
| *L. carnosum* DSM 5576$^T$ | 7 | 40 | 2 |
| *L. citreum* KCTC 3526$^T$ | 2 | 20 | 2 |
| *L. fallax* DSM 20189$^T$ | 1 | ND | 1 |
| *L. gelidum* DSM 5578$^T$ | 17 | 100 | 2 |
| *L. lactis* KCTC 3528$^T$ | 2 | 8 | 2 |
| *L. mesenteroides* subsp. *cremoris* DSM 20346$^T$ | 24 | ND | 5 |
| *L. mesenteroides* subsp. *dextranicum* DSM 20484$^T$ | 2 | ND | 3 |
| *L. mesenteroides* subsp. *mesenteroides* KCTC 3505$^T$ | 2 | 20 | 2 |
| *L. pseudomesenteroides* DSM 20193$^T$ | 2 | 15 | 100 |
| *Weissella paramesenteroides* DSM 20288$^T$ | 1 | 8 | 2 |

ND, Not determined.

differed from *L. gelidum* strains in that the former contained significantly less $C_{16:1}$ (10%) than did the latter (19–20·5%). Similarly, our isolate and *L. carnosum* strains exhibited substantial differences in the relative amounts of $C_{17\text{-cyclo}}$ and $C_{19\text{-cyclo}}$. It is evident from the fatty acid analysis that our isolate belongs to the genus *Leuconostoc* and has a distinctive profile that can be used to differentiate it from other leuconostocs. Such an identification at the genus level was confirmed by 16S rDNA analysis, for which almost-complete 16S rDNA sequences were determined for strain IH25$^T$, *L. argentinum* DSM 8581$^T$, *L. citreum* KCTC 3526$^T$ and *L. gelidum* DSM 5578$^T$ (positions 28–1524, 1505 nt).

The unrooted tree based on the neighbour-joining method clearly placed strain IH25$^T$ in a clade corresponding to the genus *Leuconostoc* (Fig. 1). An identical tree topology was recovered using the Fitch–Margoliash and maximum-parsimony methods. The isolate formed a monophyletic clade with the type strains of *L. carnosum* and *L. gelidum* that was supported by a relatively high bootstrap value of 79%. The test strain showed a high level of 16S rDNA similarity to *Leuconostoc* species, ranging from 92·5% (*L. fallax* DSM 20189$^T$) to 98·9% (*L. gelidum* DSM 5578$^T$). Intermediate values were 98·3% (*L. citreum* KCTC 3526$^T$), 98·3% (*L. carnosum* NCFB 2776$^T$),

***Table 2.*** Phenotypic characteristics of *Leuconostoc* species

Taxa are labelled as: 1, strain IH25$^T$; 2, *L. argentinum*; 3, *L. carnosum*; 4, *L. citreum* KCTC 3526$^T$; 5, *L. gelidum* DSM 5578$^T$; 6, *L. lactis* KCTC 3528$^T$; 7, *L. mesenteroides* subsp. *mesenteroides*; 8, *L. pseudomesenteroides*. Data for taxa 2, 3, 7 and 8 were taken from previous studies (Dicks *et al.*, 1993; Shaw & Harding, 1989; Farrow *et al.*, 1989; Garvie, 1986). Characteristics are scored as: +, more than 90% of strains positive; −, more than 90% of strains negative; V, variable; D, delayed reaction. ND, Not determined.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Acid produced from: | | | | | | | | |
| L-Arabinose | − | V | − | + | + | + | + | V |
| Cellobiose | + | V | − | + | + | − | D | V |
| Galactose | + | + | − | + | − | + | + | + |
| Gluconate | + | − | + | + | + | − | ND | V |
| Lactose | + | + | − | − | − | + | D | V |
| Mannitol | + | V | + | + | + | − | D | − |
| Melibiose | − | + | V | − | + | − | D | + |
| Raffinose | − | + | − | − | + | − | D | + |
| Ribose | + | − | V | − | + | − | + | + |
| Salicin | + | − | − | + | + | − | D | V |
| Trehalose | + | V | + | + | + | − | + | + |
| D-Xylose | − | V | − | − | + | − | D | + |

98·2% (*L. pseudomesenteroides* NCDO 768$^T$), 97·7% (*L. mesenteroides* subsp. *mesenteroides* DSM 20343$^T$), 97·6% (*L. argentinum* DSM 8581$^T$) and 97·5% (*L. lactis* DSM 20202$^T$). All leuconostocs except *L. fallax* showed at least 97% similarity to the kimchi isolate, the level proposed as the borderline for defining bacterial genomic species on the basis of 16S rDNA comparison (Stackebrandt & Goebel, 1994). The genealogical relatedness of our isolate to other leuconostocs was elucidated further using DNA–DNA pairing experiments (Table 1). Strain IH25$^T$ showed similarity of 1–24% to the type strains of the genus *Leuconostoc* when DNA from strain IH25$^T$ was labelled. Similarly, when other strains were labelled, the highest binding, 40%, was recorded for *L. gelidum* DSM 5578$^T$. In any case, the homology values were less than 70%, which has been proposed to define genomic species (Wayne *et al.*, 1987). It is evident from 16S rDNA and DNA–DNA hybridization data that strain IH25$^T$ represents an independent genomic species that can be separated genetically from all validly described leuconostocs. The combination of tests for acid production from sugars readily distinguishes strain IH25$^T$ from related leuconostocs (Table 2). All known strains of *L. gelidum* utilize arabinose, melibiose, raffinose and D-xylose but not galactose or lactose (Shaw & Harding, 1989). However, IH25$^T$ did not produce acid from arabinose, melibiose, raffinose or D-xylose but did produce acid from galactose and lactose. IH25$^T$ was also distinguished from strains of *L. citreum* in its usage of lactose and ribose (Table 2; Farrow *et al.*, 1989).

Leuconostocs have long been known to be residents of the normal flora of vegetable and fermented-vegetable products (Stiles, 1996). The predominant *Leuconostoc* species in kimchi is thought to be *L. mesenteroides*, although several other leuconostocs such as *L. pseudomesenteroides* and *L. lactis* are also recovered in kimchi on a regular basis. Our recent work has shown that *L. citreum* was one of the predominant species during kimchi fermentation at 20 °C and that *L. gelidum* was a dominant species at lower temperatures, for example 10 °C (J. Kim and others, unpublished results). The recognition of a new leuconostoc, i.e. strain IH25$^T$, in kimchi fermentation made from Chinese cabbage strongly implies that the microbial diversity in kimchi fermentation has been rather underestimated. A polyphasic approach is essential to characterize further the microbial diversity of kimchi fermentation.

On the basis of molecular, chemical and phenotypic data, we propose that kimchi isolate IH25$^T$ be classified in the genus *Leuconostoc* as *Leuconostoc kimchii* sp. nov.

### Description of *Leuconostoc kimchii* sp. nov.

*Leuconostoc kimchii* (kim′chi.i. M.L. gen. *kimchii* of kimchi, a traditional Korean food made by fermentation of Chinese cabbage).

Cells are spherical but similar to coccobacilli and occur singly or in pairs. Gram-positive, non-motile and non-spore-forming cells are catalase-negative and facultatively anaerobic. Grows at 15 and 37 °C but not at 45 °C. Grows in the presence of 7% NaCl but not 10% NaCl. Produces dextran from sucrose and gas from glucose. More than 95% of the lactate produced is as the D-(−) isomer. Arginine is not hydrolysed. Acid is produced from amygdalin, arabinose, cellobiose, galactose, gluconate, lactose, mannitol, ribose, salicin, trehalose, turanose, fructose, maltose, mannose and sucrose, but not from melezitose, sorbitol, starch, rhamnose, inulin, melibiose, raffinose or xylose. Major cellular fatty acids are straight-chain saturated, monounsaturated and cyclopropane-ring acids.

The G+C content of the DNA of the type strain is 37 mol%. The type and only strain, IH25$^T$, has been deposited in the Korean Collection for Type Cultures as KCTC 2386$^T$ and the culture collection of the Institute of Microbiology, Seoul National University as IMSNU 11154$^T$.

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