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

Jeongho Kim,1 Jongsik Chun2 and Hong-Ui Han1

Author for correspondence: Jeongho Kim. Tel: +82 32 860 8691. Fax: +82 32 874 6737.
e-mail: jhokim@inha.ac.kr

A Gram-positive, catalase-negative, facultatively anaerobic, coccus-shaped bacterium, designated IH25T, 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 IH25T showed the highest homology to those of Leuconostoc gelidum DSM 5578T (98.9%) and Leuconostoc citreum KCTC 3526T (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 IH25T of less than 70%. In this work, it is proposed that isolate IH25T be classified in the genus Leuconostoc as Leuconostoc kimchii sp. nov. The type strain of Leuconostoc kimchii is IH25T (= KCTC 2386T = IMSNU 11154T).

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

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**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...
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 kimichi* 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 IH25T, 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 5881T, *L. carnosum* DSM 5576T, *L. citreum* KCTC 3526T, *L. fallax* DSM 20189T, *L. gelidum* DSM 5578T, *L. mesenteroides* subsp. *mesenteroides* KCTC 3505T, *L. lactis* KCTC 3528T and *L. pseudomesenteroides* DSM 20193T. 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 H-1-l-lactic acid, Boehringer Mannheim) (Okada et al., 1978). Arginine dihydrolase activity was tested by using 1% t-arginine monohydrate chloride (Snibert & Krieg, 1994).

**Cellular fatty acid analysis.** Fatty acid methyl esters were prepared from biomass that was scraped from MRS agar plates after incubation 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 (OD600 = 0.4) in 3 ml MRS broth containing 1% (w/v) glycine were pelleted and resuspended in 380 µl 67% 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 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’-AGAAGGAGGTGATCCAGGC-3’ (positions 1525-1544) (Broius et al., 1978; Suzuki & Yamasato, 1994). PCR products were cloned into pGEM vector (Promega) and sequenced by using a dyeosequencing kit (Sequenase version 2.0, USB).

**Phylogenetic analysis.** The resultant 16S rDNA sequence of strain IH25T 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 x SSC) at 42 °C and subsequently washed twice with secondary wash solution (2 x SSC). Detection reagents were added to the membrane blots for 1 min at room temperature and then were drained off. Membrane blots were wrapped in SaranWrap 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 IH25T showed phenotypic characteristics typical of leuconostocs, i.e. a Gram-positive, non-sporoforming, 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 IH25T 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: C16:0 (35%), C19:cy9o (29%), C18:1 (16%), C16:1 (10%) and C14:0 (7%). The presence of large amounts of the C19 cyclopropane-ring acid and its precursor (C8:1) 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 IH25T

J. Kim, J. Chun and H.-U. Han
New *Leuconostoc* species from kimchi

![Fig. 1. Unrooted neighbour-joining tree based on nearly complete 16S rDNA sequences showing relationships between strain IH25T 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.](image)

**Table 1.** Mean levels of DNA relatedness (%) of strain IH25T to *Leuconostoc* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Labelled strain</th>
<th>IH25T</th>
<th>DSM 5578T</th>
<th>DSM 20193T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain IH25T</td>
<td></td>
<td>100</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td><em>L. argentinum</em> DSM 8581T</td>
<td></td>
<td>7</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><em>L. carnosum</em> DSM 5576T</td>
<td></td>
<td>7</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td><em>L. citreum</em> KCTC 3526T</td>
<td></td>
<td>2</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td><em>L. fallax</em> DSM 20189T</td>
<td></td>
<td>1</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td><em>L. gelidum</em> DSM 5578T</td>
<td></td>
<td>17</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td><em>L. lactis</em> KCTC 3528T</td>
<td></td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><em>L. mesenteroides</em> subsp. <em>cريمорис</em> DSM 20346T</td>
<td></td>
<td>24</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td><em>L. mesenteroides</em> subsp. <em>dextranicum</em> DSM 20484T</td>
<td></td>
<td>2</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td><em>L. mesenteroides</em> subsp. <em>mesenteroides</em> KCTC 3505T</td>
<td></td>
<td>2</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td><em>L. pseudomesenteroides</em> DSM 20193T</td>
<td></td>
<td>2</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Weissella <em>paramesenteroides</em> DSM 20288T</td>
<td></td>
<td>1</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

ND, Not determined.

differed from *L. gelidum* strains in that the former contained significantly less C<sub>16:1</sub> (10%) than did the latter (19–20.5%). Similarly, our isolate and *L. carnosum* strains exhibited substantial differences in the relative amounts of C<sub>17-cyclo</sub> and C<sub>19-cyclo</sub>. 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 IH25T, *L. argentinum* DSM 8581T, *L. citreum* KCTC 3526T and *L. gelidum* DSM 5578T (positions 28–1524, 1505 nt).

The unrooted tree based on the neighbour-joining method clearly placed strain IH25T 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 20189T) to 98.9% (*L. gelidum* DSM 5578T). Intermediate values were 98.3% (*L. citreum* KCTC 3526T), 98.3% (*L. carnosum* NCFB 2776T),
Table 2. Phenotypic characteristics of Leuconostoc species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid produced from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>V</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>D</td>
<td>V</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>D</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>D</td>
<td>V</td>
</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>D</td>
<td>V</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>-</td>
<td>D</td>
<td></td>
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</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>D</td>
<td>V</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>D</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>D</td>
<td>+</td>
</tr>
</tbody>
</table>

98.2% (L. pseudomesenteroides NCDO 768T), 97.7% (L. mesenteroides subsp. mesenteroides DSM 20343T), 97.6% (L. argentinum DSM 8581T) and 97.5% (L. lactis DSM 20202T). 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 IH25T showed similarity of 1–24% to the type strains of the genus Leuconostoc when DNA from strain IH25T was labelled. Similarly, when other strains were labelled, the highest binding, 40%, was recorded for L. gelidum DSM 5578T. 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 IH25T 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 IH25T 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, IH25T did not produce acid from arabinose, melibiose, raffinose or d-xylose but did produce acid from galactose and lactose. IH25T 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 IH25T, 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 IH25T 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 cocacobacilli 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, rhhamnose, inulin, melibiose, raffinose or xylose. Major cellular fatty acids are straight-chain saturated, mono-unsaturated and cyclopropane-ring acids.

The G+C content of the DNA of the type strain is 37 mol%. The type and only strain, IH25T, has been deposited in the Korean Collection for Type Cultures as KCTC 2386T and the culture collection of the Institute of Microbiology, Seoul National University as IMSNU 11154T.

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