Leuconostoc inhae sp. nov., a lactic acid bacterium isolated from kimchi

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Six strains of a hitherto unknown bacterium isolated from kimchi, a fermented vegetable food produced in Korea, were characterized by using phenotypic methods, phylogenetic analysis and DNA–DNA hybridization. The novel strains were Gram-positive, non-spore-forming, heterofermentative and spherical or lenticular lactic acid bacteria. Comparative 16S rRNA gene sequencing and DNA relatedness demonstrated that the unknown strains represented a novel clade within the genus *Leuconostoc* and were close to, but distinct from, *Leuconostoc gelidum*. The unknown strains were clearly distinguished from all described members of the genus *Leuconostoc* by using RFLP patterns of genus-specific 16S rRNA gene PCR products with a single endonuclease, *Bsm*AI. Based on the polyphasic evidence, the unknown isolates are classified as *Leuconostoc inhae* sp. nov. The type strain is strain IH003^T (=KCTC 3774^T=DSM 15101^T).

Kimchi is a traditional Korean food prepared from various vegetables and consumed as a main side dish. It is kept in a cryogenic storage system for between 2 weeks and 4 months, thereby allowing *leuconostocs* to predominate in the whole fermentation. We have been focusing on the diversity of species and, at present, five *Leuconostoc* species have been isolated from kimchi: *Leuconostoc gelidum*, *Leuconostoc mesenteroides*, *Leuconostoc citreum* (Kim *et al.*, 2000a), *Leuconostoc kimchii* (Kim *et al.*, 2000b) and dextran-producing *Leuconostoc lactis* (Kim *et al.*, 2001). As a part of a study of the microflora of kimchi, we have isolated six strains that fall into a group distinct from all known members of the genus *Leuconostoc* (Garvie, 1986; Shaw & Harding, 1989; Farrow *et al.*, 1989; Martinez-Murcia & Collins, 1991; Dicks *et al.*, 1993; Björkroth *et al.*, 2000; Kim *et al.*, 2000b; Antunes *et al.*, 2002), but are closely related to *L. gelidum*. In this article, we report the characterization of these unknown lactic acid bacteria and the results of a polyphasic taxonomy study. Based on the results, a novel species is described, *Leuconostoc inhae* sp. nov.

Six strains, IH003^T, IH101, IH201, IH316, IH515 and IH611, were isolated from kimchi fermented for 18 days at 5–8 °C. These unknown strains were cultured with 14 *Leuconostoc* and one *Weissella* species as reference type strains at 25 °C on MRS agar (Difco) or in broth. The morphology of isolates was examined by using scanning electron microscopy (S-4200; Hitachi). Additional

biochemical and physiological tests were performed according to the methods of Smibert & Krieg (1994) and Garvie (1984). Growth at different temperatures and pH and tolerance of NaCl were tested in MRS broth. Carbohydrate fermentation patterns were determined at 25 °C by using API CH50 strips and the API CHL medium system according to the manufacturer's instruction (bioMérieux). The optical isomer of lactic acid was determined by using a D-/L-lactate dehydrogenase kit (TC D-/L-lactic acid; Boehringer Mannheim).

Sequencing of 16S rDNA was carried out as described by Kim *et al.* (2000b). The 16S rDNA sequences of isolates were aligned by using CLUSTAL X software (version 1.8). Similarity values were calculated using SIMILARITY MATRIX (version 1.1) in RDP-II (Maidak *et al.*, 2001). Calculation of an evolutionary distance matrix (Kimura two-parameter model), construction of a neighbour-joining phylogenetic tree and bootstrap analysis (1000 replicates) were carried out by using PHYLIP (Felsenstein, 1993). DNA–DNA hybridization was performed as described by Kim *et al.* (2000b), using the protocol described in the ECL direct nucleic acid labelling and detection system (RPN 3000; Amersham Pharmacia Biotech). G+C contents were determined by HPLC as described by Tamaoka & Komagata (1984).

Differentiation of *Leuconostoc* species was carried out by the method described by Jang (2002). For the detection of *Leuconostoc* species, the following genus-specific primers were used to amplify 16S rRNA genes: forward primer 5'-CGAAAGGTGCTTGACCTTTCAAG-3' (*Escherichia coli* numbering system, positions 74–98); reverse primer 5'-TTTGTCTCCGAAGAGAACA-3' (positions 1023–1040).

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The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains IH003^T and IH611 are AF439560 and AY117686.

These primers produced a 976 bp PCR fragment. PCR amplification was carried out with the method described by Kim *et al.* (2000b). For species identification, restriction fragment length polymorphism (RFLP) analysis of PCR products (976 bp) was performed with four endonucleases, *Mse*I, *Hae*III, *Tsp*509I and *Bsm*AI (New England Biolabs). Digestions were performed at 37 (*Mse*I, *Hae*III), 55 (*Bsm*AI) or 65 (*Tsp*509I) °C according to the manufacturer's instructions.

Cellular morphology of the six isolates was spherical or lenticular, showing the shape typical of members of the genus *Leuconostoc* (Garvie, 1986). Cells of a representative strain IH003^T are shown in Fig. 1. They were Gram-positive, catalase-negative, arginine dihydrolase-negative and produced gas and over 92 % D-lactic acid from glucose. They grew at pH 4.8 and in 3 % NaCl and at 1, 5 and 30 °C but not at 37 °C. Dextran production from 5 % sucrose agar medium was variable (~50 %). Cellular morphology and general biochemical characteristics of the isolates were consistent with their assignment to the genus *Leuconostoc* (Garvie, 1986). The details are presented in Table 1 and in the species description below.

The 16S rRNA genes of IH003^T and IH611 were sequenced and subjected to a comparative analysis to establish the phylogenetic relationship of the isolates. The almost-complete gene sequences (1474 and 1505 nt) were determined and found to be identical. Sequence database searches showed that the sequence similarity of IH003^T and IH611 was 99.1 and 98.9 % to *L. gelidum* DSM 5578^T. The phylogenetic position of these isolates within the genus *Leuconostoc* is presented in Fig. 2. The isolates were most closely related to, but different from, *L. gelidum* DSM 5578^T, and constituted a 'cold cluster' with *L. kimchii* KCTC 2386^T, *Leuconostoc carnosum* DSM 5576^T, *Leuconostoc gasicomitatum* LMG 18811^T and *L. gelidum* DSM 5578^T, frequently isolated at low temperatures (Shaw & Harding, 1989; Kim *et al.*, 2000b; Björkroth *et al.*, 2000). The mean G + C content of the six isolates was 39.9 ± 0.5 mol%. This value is within a range

Fig. 1. Scanning electron micrograph of *Leuconostoc inhae* sp. nov. IH003^T. Cells were grown in MRS medium at 25 °C for 24 h. Bar, 2 µm.

Table 1. Differential characteristics of *Leuconostoc inhae* sp. nov. and related leuconostocs

Species/strains: 1, *L. inhae* sp. nov. (*n*=6; data from this study); 2, *L. gelidum* DSM 5578^T (data from Shaw & Harding, 1989); 3, *L. carnosum* DSM 5576^T (Shaw & Harding, 1989); 4, *L. gasicomitatum* LMG 18811^T (Björkroth *et al.*, 2000); 5, *L. kimchii* KCTC 2386^T (Kim *et al.*, 2000b). Results are scored as: +, ≥90 % strains positive; −, ≥90 % strains negative; d, 11–89 % strains positive (number of strains positive is shown). All strains/species showed the following results for acid production: positive for D-fructose, D-glucose, D-mannose, methyl α-D-glucoside, ribose, sucrose and trehalose; negative for D-arabinose, dulcitol, erythritol, glycerol, melezitose, rhamnose, sorbitol and L-sorbose. Other characteristics are mentioned in the species description.

Acid produced from:	1	2	3	4	5
Amygdalin	d (3/6)	+	−	−	+
L-Arabinose	+	+	−	+	+
Arbutin	−	+	−	−	−
Cellobiose	+	+	−	+	+
Galactose	d (3/6)	−	−	+	+
Gluconate	d (3/6)	−	−	+	+
Lactose	−	−	−	−	+
Mannitol	+	−	−	−	+
Maltose	+	−	−	+	+
Melibiose	−	+	−	+	−
Raffinose	−	+	−	+	−
Salicin	d (3/6)	+	−	−	+
D-Xylose	−	+	−	+	−

for members of the genus *Leuconostoc* (38–44 mol%; Garvie, 1986). The isolates showed more than 73 % DNA relatedness to each other but less than 26 % to all reference strains used (Table 2). Thus, the isolates were considered to represent a novel species belonging to *Leuconostoc*.

However, the separation of the isolates from *L. gelidum* was not definite, since they formed a phylogenetically close branch and had high sequence similarity, as mentioned above. We applied a PCR-RFLP method (Jang, 2002) by using a set of *Leuconostoc* genus-specific primers present in the 16S rRNA gene. The resultant PCR products were subjected to cascade digestion with three restriction enzymes, *Mse*I, *Hae*III and *Tsp*509I, yielding different combined PCR-RFLP patterns and enabling not only their detection but also identification at the genus and species level. Jang (2002) demonstrated that all described members of the genus *Leuconostoc* were successfully distinguished by RFLP patterns, excluding phylogenetically quite distant species such as *Leuconostoc fallax*, *Leuconostoc ficulneum* and *Leuconostoc fructosum* (Antunes *et al.*, 2002; Martinez-Murcia & Collins, 1991). Nevertheless, our isolates could not be differentiated with these enzymes, since they showed identical RFLP patterns to *L. gelidum* DSM 5578^T (data not shown). *Bsm*AI was chosen theoretically from a highly variable region of the 16S rRNA gene (positions 183–207) to

Fig. 2. Unrooted phylogenetic tree based on 16S rDNA comparisons showing the relationships of *L. inhae* sp. nov. IH003^T and IH611 to other *Leuconostoc* strains. Bootstrap percentages obtained with 1000 resamplings are given at branch points. Accession numbers are given in parentheses. Bar, genetic distance of 0.01.

allow separation between the novel isolates and *L. gelidum* DSM 5578^T by using WWWTacg version 2.38 (<http://genzi.virus.kyoto-u.ac.jp/tacg2/tacg2.form.html>). Experimental RFLP patterns are shown in Fig. 3. *L. kimchii* KCTC 2386^T, *L. carnosum* DSM 5576^T, *L. gasicomitatum* LMG 18811^T and *L. gelidum* DSM 5578^T (a 'cold cluster') revealed identical RFLP patterns (fragment sizes 232 and 711 bp), but the pattern differed from that of the six novel isolates (fragment sizes 107, 125 and 711 bp). Interestingly, RFLP patterns with *Bsm*AI appeared to be identical in all *Leuconostoc* species with the exception of the novel isolates (data not shown). This indicates that the isolates can be differentiated independently with a single restriction enzyme, *Bsm*AI, without using three enzymes as suggested above. PCR-RFLP analyses confirmed the phylogenetic homogeneity of the six isolates and their separation from other species of the genus *Leuconostoc*. These results are also supported by RFLP as a good distinct method for strain

characterization (Heyndrickx *et al.*, 1996). Thus, based on evidence from cellular morphology, phylogenetic analysis, DNA relatedness and PCR-RFLP patterns and their distinctive biochemical characteristics, the six isolates from kimchi are assigned to the genus *Leuconostoc* as *Leuconostoc inhae* sp. nov.

Description of *Leuconostoc inhae* sp. nov.

Leuconostoc inhae (in'ha.e. N.L. gen. n. *inhae* of Inha, from the Inha University, Republic of Korea).

Cells are Gram-positive, spherical or lenticular, occurring singly or in pairs. Cells are 0.8–1.0 × 0.8–1.8 µm. Colonies on MRS agar after 48 h at 25 °C are small (1.0–1.5 mm), round, smooth, convex, opaque and greyish-white. Cells are non-motile and non-spore-forming. Facultatively anaerobic. Catalase-negative. Obligately heterofermentative. Arginine is not hydrolysed. Some strains produce slime

Table 2. Levels of DNA relatedness among *L. inhae* sp. nov. and *Leuconostoc* species within the same cluster

Percentage reassociation is shown. Reassociation values are means of duplicate determinations.

Source of unlabelled DNA	Source of labelled DNA	
	<i>L. inhae</i> IH003 ^T	<i>L. gelidum</i> DSM 5578 ^T
<i>L. inhae</i>		
IH003 ^T	100	11
IH101	78	22
IH201	99	19
IH316	89	10
IH515	73	12
IH611	120	16
<i>L. carnosum</i> DSM 5576 ^T	20	2
<i>L. gasicomitatum</i> LMG 18811 ^T	20	6
<i>L. gelidum</i>		
DSM 5578 ^T	26	100
IH619	25	97
<i>L. kimchii</i> KCTC 2386 ^T	23	4

Fig. 3. PCR-RFLP patterns of *L. inhae* sp. nov. and closely related species derived from digestion of genus-specific PCR products with *Bsm*AI. Lanes: 1, *L. carnosum* DSM 5576^T; 2, *L. gasi-comitatum* LMG 18811^T; 3, *L. gelidum* DSM 5578^T; 4, *L. kimchii* KCTC 2386^T; 5–10, *L. inhae* sp. nov. IH003^T, IH101, IH201, IH316, IH515 and IH611; M, 100 bp DNA ladder. RFLP patterns with *Bsm*AI were identical in all strains of *Leuconostoc* examined except *L. inhae* strains and the phylogenetically distant strains *L. fallax* DSM 20189^T, *L. ficulneum* DSM 13613^T and *L. fructosum* DSM 20349^T (not shown). Only four species in the same cluster as *L. inhae* sp. nov. are shown.

materials. Growth occurs in 3 % NaCl but not in 7 % NaCl and at pH 4–8 but not at pH 3–8. All strains grow at 30 °C but not at 37 °C. Good growth is obtained at 1–5 °C. Over 92 % D-lactic acid is produced from glucose. Acid is produced from L-arabinose, cellobiose, aesculin, D-fructose, β -gentiobiose, D-glucose, D-mannose, mannitol, maltose, methyl α -D-glucoside, N-acetylglucosamine, ribose, sucrose and trehalose. Acid is not produced from adonitol, D-arabinose, D-arabitol, arbutin, 2-ketogluconate, 5-ketogluconate, dulcitol, erythritol, D-fucose, L-fucose, glycerol, glycogen, inositol, inulin, lactose, D-lyxose, melezitose, melibiose, methyl α -D-mannoside, methyl β -xyloside, D-raffinose, rhamnose, sorbitol, L-sorbose, starch, D-tagatose, xylitol, D-xylose or L-xylose. Some strains produce acid from amygdalin, galactose, gluconate, salicin and D-turanose. The G+C content of the type strain is 39.9 ± 0.5 mol% (as determined by HPLC).

The type strain, strain IH003^T (=KCTC 3774^T=DSM 15101^T), was isolated from kimchi. The description of the type strain corresponds to that of five other isolates, except that no growth occurs at pH 3–8 and slime material is not produced from sucrose. Amygdalin, galactose, gluconate, salicin and D-turanose are not fermented.

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