Lactobacillus senmaizukei sp. nov., isolated from Japanese pickle

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The taxonomic position of a lactic acid bacterium, strain L13^T, isolated from senmaizuke, a Japanese traditional pickle, was studied. This strain was a heterofermentative, facultatively anaerobic, Gram-positive, non-spore-forming, non-motile, rod-shaped bacterium. It produced DL-lactic acid from glucose. Phylogenetic analysis of its 16S rRNA gene sequence and physiological and biochemical characteristics indicated that the strain was a member of the genus *Lactobacillus*. Based on 16S rRNA gene sequence similarity, strain L13^T was closely related to *Lactobacillus hammesii* CIP 108387^T (98.9%) and *Lactobacillus parabrevis* ATCC 53295^T (98.5%). In addition, the *rpoA* gene sequence of strain L13^T was closely related to those of *L. hammesii* CIP 108387^T (92.2%) and *L. parabrevis* ATCC 53295^T (92.0%). However, DNA–DNA hybridization of strain L13^T with these two strains revealed that strain L13^T represents a separate genomic species. The DNA G+C content of strain L13^T was 46 mol% and the peptidoglycan was of the A4 α L-Lys–D-Asp type. Thus, these data indicate that strain L13^T represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus senmaizukei* is proposed. The type strain is L13^T (=NBRC 103853^T=TISTR 1847^T).

Japan, like many other countries, has a long tradition of producing pickles. Pickles can be used as good screening sources for the isolation of valuable micro-organisms, e.g. lactic acid bacteria. Recently, a bacterium, strain L13^T, was isolated from senmaizuke, one of the traditional fermented pickles made from Japanese radish in Kyoto, Japan (Ueno *et al.*, 2007). In this study, strain L13^T was assessed using phenotypic and chemotaxonomic characteristics, 16S rRNA and RNA polymerase alpha subunit (*rpoA*) gene sequence analyses and DNA–DNA relatedness studies. The results showed that strain L13^T represents a novel species of the genus *Lactobacillus*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoA* gene sequences of strain L13^T are AB297927 and AB359074, respectively. The accession number for the *rpoA* gene sequence of *Lactobacillus parabrevis* ATCC 53295^T is AB359076.

Phylogenetic trees based on 16S rRNA gene sequences derived by the neighbour-joining (Supplementary Fig. S1), maximum-likelihood (Supplementary Fig. S2) and maximum-parsimony (Supplementary Fig. S3) methods showing the position of *Lactobacillus senmaizukei* L13^T among selected lactobacilli are available with the online version of this paper.

One hundred colonies were isolated from senmaizuke after cultivation on GYP agar plates (Ueno *et al.*, 1997) at 30 °C for 5 days. Colonies were cultivated anaerobically in GYP liquid medium containing 5% sodium glutamate at 30 °C for 48 h. The culture supernatant of each isolate was analysed for production of γ -aminobutyric acid by TLC (Ueno *et al.*, 2007); strain L13^T was obtained as a high-level producer of γ -aminobutyric acid. Cell morphology, Gram staining, colony appearance, spore formation and catalase activity were examined on GYP agar after incubation for 48 h. Sugar fermentation patterns were determined using the API 50 CH and API 50 CHL kits (bioMérieux) over a period of 72 h cultivation at 30 °C. An F-kit DL-lactic acid (Roche Diagnostics) was used to determine production of D- and L-lactic acids from glucose. The effects of various temperatures on growth were determined by using GYP broth at 15, 30 and 45 °C. Bacterial growth was monitored by measurement of OD₆₆₀. Tolerance of NaCl was examined by adding 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% (w/v) NaCl to GYP broth. Production of ammonia from arginine was determined according to the method of Abo-Elnaga & Kandler (1965). The cell wall composition was determined according to Schleifer & Kandler (1972).

DNA was isolated by using the lytic enzyme labiase according to the method of Niwa *et al.* (2005) with some modifications. Genomic DNA was extracted from 0.36 g wet weight cells as follows. After addition of 0.36 ml 0.1 M acetate buffer (pH 4.0) and 0.36 ml 4 mg labiase ml⁻¹ to the cells, the cell suspension was incubated for 10 min at 55 °C. After this incubation, 0.39 ml 20 % SDS was added and the suspension was mixed gently and incubated for 5 min at 55 °C. After addition of 2 µl 5 mg RNase solution ml⁻¹, the suspension was incubated at 37 °C for 1 h. Subsequent chloroform/isoamyl alcohol extraction, precipitation and spooling of DNA on a glass rod were performed as described by Marmur (1961). The isolated genomic DNA was used for amplification of the 16S rRNA gene by PCR using primers 27f (5'-AGAGTTTGATCCTGG-CTCAG-3') and 1492r (5'-GGCTACCTGTTCAGACTT-3') (Weisburg *et al.*, 1991). The *rpoA* gene was amplified as described by Naser *et al.* (2005). The *rpoA* gene of *Lactobacillus parabrevis* ATCC 53295^T was also amplified and analysed since it was not available in the database. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using the NJPLOT program (Perrière & Gouy, 1996) and by the maximum-likelihood (Felsenstein, 1989) and maximum-parsimony methods using MEGA version 3.1 (Kumar *et al.*, 2004). Confidence values of branches of the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. DNA–DNA hybridization was carried out as described by Ezaki *et al.* (1989) and the G + C content of the DNA was determined according to Mesbah *et al.* (1989). Metabolites from glucose were

analysed by HPLC. A Develosil RPAQUEOUS-AR-5 column (Nomura Chemical) was used for organic acid detection (detection, 210 nm; mobile phase, acetonitrile; column oven, 35 °C) and a Develosil C30-UG-5 column (Nomura Chemical) was used for ethanol detection (detection, refractive index detector; mobile phase, miliQ water; column oven, 40 °C).

Strain L13^T was characterized phenotypically and genotypically (Table 1). It was a heterofermentative, facultatively anaerobic, Gram-positive, non-spore-forming, non-motile, catalase-negative, rod-shaped bacterium. Based on 16S rRNA gene sequence similarity (Fig. 1), strain L13^T belonged to the genus *Lactobacillus* and it was closely related to *Lactobacillus hammesii* CIP 108387^T (98.9%), *L. parabrevis* ATCC 53295^T (98.5%) and *Lactobacillus brevis* LMG 6906^T (97.6%). Additional trees constructed by the neighbour-joining, maximum-likelihood and maximum-parsimony methods are available as Supplementary Figs S1, S2 and S3, respectively, in IJSEM Online. In addition, the *rpoA* gene sequence of strain L13^T showed similarities of 92.2 and 92.0% to *L. hammesii* CIP 108387^T and *L. parabrevis* ATCC 53295^T, respectively, as shown in Fig. 2. Thus, *rpoA* gene analysis also showed that strain L13^T was closely related to these strains. *L. parabrevis* ATCC 53295^T was isolated from wheat and originally deposited with the ATCC as *L. brevis* (Spiller, 1987); it has recently been reclassified as *L. parabrevis* ATCC 53295^T (Vancanneyt *et al.*, 2006). DNA–DNA relatedness values of strain L13^T to *L. parabrevis* ATCC 53295^T and *L. hammesii* CIP 108387^T were 8.3 and 6.3%, respectively. These values are below the

Table 1. Differential characteristics of strain L13^T and closely related *Lactobacillus* species

Strains: 1, L13^T; 2, *L. parabrevis* ATCC 53295^T (data from Vancanneyt *et al.*, 2006); 3, *L. hammesii* CIP 108387^T (Valcheva *et al.*, 2005). +, Positive; –, negative; w, weakly positive; ND, not determined.

Characteristic	1	2	3
NH ₃ from arginine	–	+	–
Acid production from:			
Aesculin	+	–	+
L-Arabinose	–	+	+
D-Arabitol	–	+	ND
Cellobiose	–	–	+
D-Fructose	w	+	+
D-Gluconate	w	+	ND
2-Keto-D-gluconate	w	–	ND
N-Acetylglucosamine	w	+	+
D-Glucose	w	+	+
Maltose	–	+	+
Mannitol	–	–	+
Mannose	–	–	+
D-Ribose	+	+	ND
Trehalose	–	–	+
Methyl β-xyloside	–	+	+
DNA G + C content (mol%)	46	49	52.6

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the position of *Lactobacillus senmaizukei* L13^T among selected lactobacilli. The tree was generated by the neighbour-joining method. Bootstrap percentages above 55 %, based on 1000 replications, are given at nodes. Bar, 0.02 substitutions per nucleotide position.

threshold of 70 % suggested for species delineation (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), thus indicating that strain L13^T represents a separate genomic species. The DNA G + C content of strain L13^T was 46 mol%, which is within the range (32–55 mol%) reported for other species of the genus *Lactobacillus* (Kandler & Weiss, 1986). The cell wall composition was lysine and aspartic acid, indicating peptidoglycan type A4 α L-Lys–D-Asp. Strain L13^T could not produce acid from L-arabinose, methyl β -xyloside or maltose. It could grow slowly and heterofermentatively in glucose-containing medium (GYP) at 30 °C and a small amount of DL-lactate was detected in the medium. Based on its biochemical properties, sugar fermentation patterns, DNA G + C content and DNA–DNA hybridization experiments, strain L13^T clearly differed from *L. parabrevis* ATCC 53295^T (Vancanneyt *et al.*, 2006) and *L. hammesii* CIP 108387^T (Valcheva *et al.*, 2005). Thus, strain L13^T represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus senmaizukei* is proposed.

Description of *Lactobacillus senmaizukei* sp. nov.

Lactobacillus senmaizukei (sen.mai.zu'ke.i. N.L. n. *senmaizukeum* senmaizuke, a traditional fermented pickle; N.L. gen. n. *senmaizukei* of senmaizuke, referring to the isolation of the type strain from a traditional fermented pickle produced in Kyoto, Japan).

Cells are Gram-positive, catalase-negative, non-motile, non-spore-forming rods (0.5–0.8 $\times$ 1.0–5.0 μ m). Colonies on GYP agar are circular, low-convex with entire margins and non-pigmented (1.0–2.0 mm in diameter after 2 days of growth). Can grow in up to 8.0 % NaCl and at 15 °C, but not at 45 °C. The specific growth rate in 5.0 % NaCl is 50 % (100 % growth without NaCl). Facultatively anaerobic. Produces DL-lactic acid heterofermentatively from glucose. Acetic acid and ethanol are also produced as other metabolites (HPLC analysis). Acid is produced from aesculin, D-fructose, D-galactose, D-gluconate, 2-keto-D-gluconate, N-acetylglucosamine, D-ribose and D-xylose, but

Fig. 2. Phylogenetic tree based on *rpoA* gene sequences showing the relationship between *Lactobacillus senmaizukei* L13^T and selected lactobacilli. The branching pattern was generated by the neighbour-joining method. Bootstrap percentages above 55%, based on 1000 replications, are shown at nodes. Bar, 0.05 substitutions per nucleotide positions.

not from adonitol, D-amydalin, arabinose, arabitol, arbutin, dulcitol, erythritol, cellobiose, gentiobiose, 5-keto-D-gluconate, methyl α -D-glucoside, glycogen, glycerol, inositol, inulin, lactose, D-lyxose, maltose, D-mannitol, D-mannose, methyl α -D-mannoside, melezitose, melibiose, raffinose, L-rhamnose, salicin, sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol, L-xylose or methyl β -xyloside. Ammonia is not produced from arginine. Peptidoglycan structure is of the A4 α L-Lys-D-Asp type.

The type strain, L13^T (=NBRC 103853^T=TISTR 1847^T), was isolated from senmaizuke, one of the traditional pickles from Kyoto, Japan. The DNA G+C content of the type strain is 46 mol%.

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