Lactobacillus nodensis sp. nov., isolated from rice bran

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Gram-positive, rod-shaped, non-motile lactic acid bacteria, strains iz4b-1^T, iz4b-2 and iz4c-1, were isolated in an attempt to study the composition of the *Lactobacillus* flora of Japanese pickles. Analysis of their 16S rRNA gene sequences revealed that the strains clustered in the *Lactobacillus alimentarius* group, and comparatively high similarities were shown to '*Lactobacillus tuccei*' CECT 5920 (97.0%), *Lactobacillus versmoldensis* KU-3^T (96.4%) and *Lactobacillus nantensis* LP33^T (94.4%). DNA–DNA hybridization assays clearly revealed that the isolates represented a novel taxon. The DNA G + C content was 40.6 mol% and the peptidoglycan type was L-Lys–D-Asp. Thus, these isolates represent a novel *Lactobacillus* species, for which the name *Lactobacillus nodensis* sp. nov. is proposed. The type strain is iz4b-1^T (=DSM 19682^T =JCM 14932^T).

Lactic acid bacteria (LAB) play important roles in vegetable fermentations by producing lactic acid and/or alcohol, which improve the taste of pickles and help to preserve them. Many kinds of LAB, such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici*, are found widely in the various stages of fermentation. We have studied the LAB flora of a Japanese homemade pickle called nuka-zuke, which is made by pickling vegetables and rice bran paste, and isolated 103 strains from 15 independent pickle samples. 16S rRNA gene sequences of all the isolates were analysed and three strains, iz4b-1^T, iz4b-2 and iz4c-1, showed low levels of sequence similarity to any other established species. Therefore, additional tests were performed to determine whether these three strains represented a novel *Lactobacillus* species.

The strains were isolated from nuka-zuke made in Utsunomiya city, Tochigi prefecture, Japan. Samples were homogenized in 0.85% NaCl solution, serially diluted and spread on MRS (Oxoid) agar plates. Plates were incubated at 30 °C for 48 h. Colonies were picked and isolates were grown routinely in MRS broth or agar and maintained at –80 °C as glycerol stocks.

Chromosomal DNA was extracted as described by Pitcher *et al.* (1989) and used as a template for 16S rRNA gene sequence amplification. Primers 8f (5'-AGAGTTTGAT-

CCTGGCTCAG-3') and 1540r (5'-AAGGAGGTGATCC-AGCCGCA-3') were used for PCR (Endo & Okada, 2005). The PCR products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Cycle sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and seven primers listed in Endo & Okada (2005). The sequences were analysed and the closest recognized relatives of the isolates were searched for in the NCBI database using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences representing the closest matches were retrieved and then aligned using the CLUSTAL W program (Thompson *et al.*, 1994). All sequences used in the reconstruction of the phylogenetic tree were approximately 1560 bp long. A distance matrix was calculated using Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were constructed with the neighbour-joining method as described by Saitou & Nei (1987) and the maximum-parsimony method as described by Felsenstein (1978) using the software package MEGA version 4.0 (Tamura *et al.*, 2007) and the maximum-likelihood method (Felsenstein, 1981) using PHYLIP version 3.67 (Felsenstein, 2007). Statistical significance of groupings was estimated by bootstrapping (1000 replicates). The maximum-parsimony and maximum-likelihood trees are available as Supplementary Figs S1 and S2 in IJSEM Online.

The G + C contents of the strains were determined by HPLC as described by Katayama-Fujimura *et al.* (1984). DNA–DNA hybridizations were carried out in triplicate as described by Ezaki *et al.* (1988, 1989). Salmon sperm DNA was used as a control. The peptidoglycan type was determined as described by Schleifer & Kandler (1972) and Schleifer (1985).

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain iz4b-1^T is AB332024.

16S rRNA gene sequence-based maximum-parsimony and maximum-likelihood trees and detailed DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Cell morphology of the strains was observed by phase-contrast microscopy. Determination of Gram reactions was performed using a Favor G 'Nissui' kit (Nissui) following the manufacturer's instructions. Sugar-fermentation patterns were assessed using the API 50 CHL system (bioMérieux) for 48 h at 30 °C according to the manufacturer's instructions after diluting the colonies with saline. Lactic acid configuration was determined using a DL-lactate test kit (Boehringer Mannheim). Catalase activity was determined by transferring fresh colonies from MRS agar to 1.5 ml tubes containing 5 % H₂O₂. The production of gas from glucose was assayed by cultivation of the bacteria in tubes covered with 2 % agar plugs above the MRS broth.

Partial 16S rRNA gene sequences (about 1560 bp) were obtained from strains iz4b-1^T, iz4b-2 and iz4c-1. Values of sequence similarity indicated that the closest neighbours of strain iz4b-1^T were '*Lactobacillus tuccei*' CECT 5920 (97.0 %), *Lactobacillus versmoldensis* KU-3^T (96.4 %) and *Lactobacillus nantensis* LP33^T (94.4 %). They belong to the *Lactobacillus alimentarius* group (Fig. 1). The sequence similarity between strain iz4b-1^T and strains iz4b-2 and iz4c-1 was 99.7 and 99.5 %, respectively.

Fig. 1. Phylogenetic tree showing the relationships between strain iz4b-1^T and related *Lactobacillus* species on the basis of 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method. Bootstrap values based on 1000 replications are listed as percentages at branching points; only values above 50 % are indicated. Bar, 0.01 K_{nuc} (indicates nucleotide substitutions per site). The *L. alimentarius* group (Felis & Dellaglio, 2007) includes *L. farciminis*, *L. crustorum*, *L. nantensis*, *L. mindensis*, *L. alimentarius*, *L. kimchii*, *L. paralimentarius*, *L. versmoldensis*, '*L. tuccei*' and *L. nodensis* sp. nov. Maximum-likelihood and maximum-parsimony trees are shown in Supplementary Figs S1 and S2.

Strains iz4b-1^T, iz4b-2 and iz4c-1 showed high levels of DNA–DNA hybridization values (90–98 %) to one another, while the type strain iz4b-1^T showed low levels of DNA–DNA relatedness to '*L. tuccei*' CECT 5920 (9, 14 and 19 %; Supplementary Table S1). The DNA G+C content of strain iz4b-1^T was 40.6 mol% and that of '*L. tuccei*' CECT 5920 was 35.9 mol%. This value is within the range reported for the genus *Lactobacillus* (32–53 mol%) by Kandler *et al.* (1983). The peptidoglycan type of strain iz4b-1^T was L-Lys–D-Asp, which is the major type in the *L. alimentarius* group (Chenoll *et al.*, 2005). Thus, we conclude that the three isolates belong to the same taxon in the *L. alimentarius* group and that the taxon is differentiated from known *Lactobacillus* species (Johnson, 1973; Stackebrandt & Goebel, 1994). We are planning additional analysis to identify differences between the three strains.

Physiological and biochemical characteristics of strain iz4b-1^T, '*Lactobacillus tuccei*' CECT 5920 and *L. versmoldensis* DSM 14857^T are listed in Table 1. Strains iz4b-1^T, iz4b-2 and iz4c-1 showed almost the same patterns of sugar fermentation with the exception of D-ribose. On the basis of phylogenetic and phenotypic results, strain iz4b-1^T is proposed as the type strain of a novel species, *Lactobacillus nodensis* sp. nov.

Description of *Lactobacillus nodensis* sp. nov.

Lactobacillus nodensis (no.den'sis. N.L. masc. adj. *nodensis* referring to Noda, the city in which the bacterium was originally isolated).

Cells are Gram-positive, non-motile, non-spore-forming rods, 1.8 × 5 µm in size, occurring singly or in pairs. After anaerobic growth at 30 °C for 48 h, colonies on MRS agar are 1 mm in diameter, round with rough surfaces. Catalase-negative. Obligately homofermentative; gas is not produced from glucose. Both D- and L-lactic acid are produced from glucose. Growth occurs at 15–37 °C but not at 45 °C. Growth occurs at pH 4.0 but not at pH 2.6. Grows in the presence of up to 15 % NaCl in MRS broth. Acid is produced from D-arabinose, D-ribose (strain-dependent weak reaction), D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine and aesculin. No acid is produced from erythritol, L-arabinose, DL-xylose, adonitol, methyl β-D-xyloside, sorbose, rhamnose, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, gentiobiose, turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, glycerol, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, inulin, starch, glycogen, xylitol, gluconate, 2-ketogluconate or 5-ketogluconate. The DNA G+C content of the type strain is 40.6 mol%. The peptidoglycan is of the L-Lys–D-Asp type.

The type strain is strain iz4b-1^T (=DSM 19682^T =JCM 14932^T). The type strain and reference strains iz4b-2 and iz4c-1 were isolated from Japanese pickles collected in Utsunomiya city, Tochigi prefecture, Japan.

Table 1. Phenotypic characteristics of strain iz4b-1^T and closely related species in the *L. alimentarius* group

Strains: 1, *L. nodensis* sp. nov. iz4b-1^T (this study); 2, '*L. tuceti*' CECT 5920 (Chenoll *et al.*, 2005); 3, *L. versmoldensis* KU-3^T (Kröckel *et al.*, 2003); 4, *L. kimchii* MT-1077^T (Yoon *et al.*, 2000); 5, *L. paralimentarius* TB 1^T (Cai *et al.*, 1999); 6, *L. farciminis* DSM 20184^T (Reuter, 1983); 7, *L. alimentarius* DSM 20249^T (Reuter, 1983); 8, *L. mindensis* TMW 1.80^T (Ehrmann *et al.*, 2003); 9, *L. crustorum* LMG 23699^T (Scheirlinck *et al.*, 2007); 10, *L. nantensis* LP33^T (Valcheva *et al.*, 2006). +, Positive; –, negative; w, weak reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Growth at/in:										
15 °C	+	–	+	+	ND	+	+	+	+	+
45 °C	–	–	–	–	–	–	–	–	+	–
10 % NaCl	+	–	+	–	–	+	–	ND	–	–
Isomeric type of lactate	D, L	D, L	L (D)	D, L	L (D)	L (D)	D, L	D, L	D, L	D, L
Acid production from:										
D-Arabinose	+	–	–	–	–	+	–	–	–	–
D-Ribose	w*	+	+	+	+	+	+	–	–	w
D-Mannose	+	+	–	–	ND	ND	+	+	+	+
Rhamnose	–	+	–	–	+	+	–	+	–	–
D-Mannitol	–	+	–	–	–	–	–	–	–	+
Maltose	–	+	+	+	+	w	+	w	+	+
D-Fucose	–	+	ND	–	–	ND	–	–	–	–
Melibiose	–	–	+	–	–	–	–	–	–	–
Peptidoglycan type	L-Lys– D-Asp	L-Lys–Gly– D-Asp	L-Lys– D-Asp	L-Lys– D-Asp	L-Lys– D-Asp	L-Lys– D-Asp	L-Lys– D-Asp	L-Lys– D-Asp	ND	L-Lys– D-Asp

*Strain iz4c-1 was negative for D-ribose.

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