Lactobacillus furfuricola sp. nov., isolated from Nukadoko, rice bran paste for Japanese pickles

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Two strains of lactic acid bacteria, Nu27^T and Nu29, were isolated from Nukadoko, rice bran paste for Japanese pickles. The isolates were Gram-stain-positive, rod-shaped, catalase-negative, non-motile and facultatively anaerobic lactic acid bacteria. The isolates showed identical 16S rRNA gene sequences. The closest relatives to strain Nu27^T based on 16S rRNA gene sequence similarities were *Lactobacillus versmoldensis* KU-3^T (98.9 % 16S rRNA gene sequence similarity), *Lactobacillus nodensis* iz4b^T (96.3 %) and *Lactobacillus tucceti* CECT 5290^T (97.2 %). DNA–DNA relatedness values revealed genotype separation of the two isolates from the above three species. Based on the physiological, biochemical and genotypic characteristics provided, the isolates represent a novel species of the genus *Lactobacillus*, for which name is *Lactobacillus furfuricola* proposed. The type strain is Nu 27^T (=JCM 18764^T=NRIC 0900^T=DSM 27174^T).

There are many fermented foods in Japan. Lactic acid bacteria (LAB) play important roles in several fermented foods, such as improvement of taste and safe fermentation. Nukaduke is one of the traditional fermented foods that are widely produced in Japan. Nukaduke is made by fresh vegetables pickling in rice bran paste called Nukadoko. Nukadoko is prepared by natural fermentation of rice bran mixed with salt-water; it is maintained by agitation and adding fresh rice bran into Nukadoko.

Nukadoko contains many kinds of LAB such as *Lactobacillus acetotolerans*, *Lactobacillus acidifarinae*, *Lactobacillus versmoldensis*, *Leuconostoc mesenteroides* (Nakayama *et al.*, 2007). The bacterial microbiota of Nukadoko was investigated by pyrosequencing of 16S rRNA gene (Sakamoto *et al.*, 2011). According to the study, 44 species of the genus *Lactobacillus* were detected from the 16 samples. This result shows that the lactic acid bacterial community structure in Nukadoko was very diverse. However, only some LAB occurring in Nukadoko can be cultivated.

During the study of the lactic acid bacterial biota in Nukadoko, 50 strains were isolated from seven Nukadoko samples in Japan. Based on partial sequences of the 16S rRNA gene, strains Nu27^T and Nu29 formed a subcluster in the *Lactobacillus alimentarius–Lactobacillus farciminis* phylogenetic group, and were closely related to type strains of *Lactobacillus versmoldensis*, *Lactobacillus nodensis* and *Lactobacillus tucceti*. Based on DNA−DNA relatedness values, the two isolates are genetically distinct from the phylogenetic relatives. Here we describe the taxonomic characteristics of the isolates and propose a novel species of the genus *Lactobacillus*.

The enrichment culture approach was employed for the isolation of LAB from Nukadoko. Sake broth containing 10 p.p.m. sodium azide and 10 p.p.m. cycloheximide was used for enrichment culture. Sake medium comprised (l^{-1} distilled water): 2.0 g glucose, 1.0 g peptone, 1.0 g yeast extract, 3.0 g Lab-Lemco (Oxoid) and 300 ml Japanese sake; the pH was adjusted to 6.0 with HCl. After autoclaving, the media was cooled to room temperature and 50 ml ethanol was added. Samples were inoculated into the

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Lactobacillus furfuricola* Nu27^T and Nu29 are AB910349 and AB910350, respectively. The GenBank/EMBL/DDBJ accession numbers for the *pheS* sequences of *Lactobacillus furfuricola* Nu27^T and Nu29, *Lactobacillus nodensis* JCM 14932^T and *Lactobacillus tucceti* JCM 18037^T are AB910351, AB910352, AB910353 and AB910354, respectively, and those for the *rpoA* sequences of *Lactobacillus furfuricola* Nu27^T and Nu29, *Lactobacillus nodensis* JCM 14932^T and *Lactobacillus tucceti* JCM 18037^T are AB910355, AB910356, AB910357 andAB910358, respectively.

Six supplementary figures and a supplementary table are available with the online version of this paper.

broth and incubated at 30 °C. After 3 or 5 days, the cultured broth was mixed into MRS medium with 0.6 % agar containing 10 p.p.m. sodium azide and 10 p.p.m. cycloheximide and incubated at 30 °C for 3 days. Colonies were picked from the MRS agar medium. Isolates were maintained on MRS agar containing 1.0 % $CaCO_3$ and at −80 °C as glycerol stocks. *Lactobacillus nodensis* JCM 14932^T, *Lactobacillus tucceti* JCM 18037^T and *Lactobacillus versmoldensis* JCM 16174^T were used as reference strains in this study and were cultured in the MRS medium.

Chromosomal DNA was prepared from bacterial strains and was then used as a template for amplification of the 16S rRNA gene, phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) gene sequences. Amplification of 16S rRNA gene sequences and purification of PCR products were performed according to the method described previously (Irisawa & Okada 2009). The *pheS* and *rpoA* sequences of strains Nu27^T, Nu29, *Lactobacillus nodensis* JCM 14932^T and *Lactobacillus tucceti* JCM 18037^T were amplified by PCR with following primer sets; pheS-21-F (5′-CAYCCNGCHCGYGAYATGC-3′)/ pheS-22-R (5′-CCWARVCCRAARGCAAARCC-3′) and rpoA-21-F (5′-ATGATYGARTTTGAAAAACC-3′)/rpoA-23-R (5′-ACHGTRTTRATDCCDGCRCG-3′) (Naser *et al.*, 2005). Amplification of these gene sequences and purification of the PCR products were performed according to the method described previously (Tohno *et al.*, 2013). PCR products were sequenced using Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) and a model 3130 Genetic Analyzer (ABI PRISM) automatic DNA sequencer. The closest recognized relatives of the isolates were determined by performing database searches and each gene sequence of closely related species were obtained from the DDBJ database. These sequences were aligned using CLUSTAL X (version 2.1) (Larkin *et al.*, 2007). A distance matrix was obtained by the two-parameter method of Kimura (1980). The neighbour-joining method was used to reconstruct a phylogenetic tree (Saitou & Nei, 1987). The robustness of the individual branches of the tree was established by bootstrapping with 1000 replicates (Felsenstein, 1985). Maximum-likelihood analysis was also performed using version 3.0 of the PhyML program (Guindon & Gascuel, 2003) and optimal models of nucleotide substitution were estimated with jModelTest version 2.1.4 (Darriba *et al.* 2012) using the Akaike information criterion (AIC); the models selected were GTR + I + G for the 16S rRNA gene, TVM + I + G for *pheS* and TIM2 + I + G for *rpoA*. Approximately 1500 nt of the 16S rRNA gene, 430 nt of the *pheS* and 800 nt of the *ropA* sequences were determined and compared to other nucleotide sequences in the DDBJ, EMBL and GenBank databases.

Based on the 16S rRNA gene sequences, strains Nu27^T and Nu29 showed high sequence similarities (100 %) with each other. The highest 16S rRNA gene sequence similarities of strains Nu27^T and Nu29 were with *Lactobacillus versmoldensis* KU-3^T *Lactobacillus nodensis* iz4b^T and *Lactobacillus tucceti* CECT 5290^T, the values being 98.9, 96.3 and 97.2 %, respectively. Based on the *pheS* and *rpoA* analysis, the sequence similarities between Nu27^T and Nu29 were 98.6 and 99.3 %, respectively. A lower *pheS* and *rpoA* sequence similarity of <89.3 % with closely related species was found for the two strains. For the reconstruction of phylogenetic trees, 1392 nt of the 16S rRNA gene, 304 nt of the *pheS* and 356 nt of the *rpoA* sequences were used. The phylogenetic tree was evaluated by using bootstrap analysis with 1000 replicates. Both neighbour-joining and maximum-likelihood trees showed that the isolates formed a different cluster with closely related species (Fig. 1 and Figs S1, S2, S3, S4 and S5, available in the online Supplementary Material). Based on the phylogenetic analysis, we concluded that the two isolates are located in the *Lactobacillus alimentarius–Lactobacillus farciminis* group. Furthermore, in the genus *Lactobacillus*, interspecies gaps based on *pheS* and *rpoA* gene sequences exceed 10 % and 5 %, respectively (Naser *et al.*, 2007). In this study, the *pheS* and *rpoA* sequence similarities of the two isolates with closely related species were less than 83.4 % and 89.3 %, respectively, confirming that the two isolates are distinct species of the genus *Lactobacillus*.

Extraction of bacterial DNA was performed by using a Qiagen Genomic-tip 100/G kit (Qiagen) following the manufacturer's instructions. DNA–DNA hybridization was carried out by the technique of fluorometric hybridization in microdilution wells (Ezaki *et al.*, 1989). The DNA G + C contents of the isolates were determined by HPLC as described by Tamaoka & Komagata (1984) and Kitahara *et al.* (2001). The DNA–DNA relatedness value was high between the two isolates (>92.6 %). In contrast, the DNA–DNA relatedness values of the isolates to *Lactobacillus versmoldensis* JCM 16174^T (<14.0 %), *Lactobacillus nodensis* JCM 14932^T (<10.6 %) and *Lactobacillus tucceti* JCM 18037^T (<14.9 %) were low. The DNA G + C contents of strains Nu27^T and Nu29 were 40.0 and 40.8 mol%, respectively.

To differentiate the two isolates, randomly amplified polymorphic DNA (RAPD) fingerprinting was performed according to the method of Endo & Okada (2006). Primer D (5′-GGCCACGGAA-3′) and primer F (5′-GGCCACGGAA-3′) were used in this study. The DNA fingerprints indicated that the two strains were genetically similar, but were different at the strain level (Fig. S6).

Cell size, cell form and the Gram stain were examined on cells grown in MRS broth at 30 °C for 48 h. Catalase was detected by the addition of 3 % (w/v) H_2O_2 on the MRS agar plate. Motility was tested in MRS soft agar. Gas production from glucose was determined using a Durham tube in MRS broth. The effect of temperature (4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C) and different initial pH (3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 8.5, 9.0) were tested by using MRS broth. Tolerance to NaCl was examined in MRS containing 0–20 % (w/v) NaCl after incubation for 5 days at 30 °C.

The type of fermentation was determined using an F-kit ethanol (Roche Diagnostic) as described by Okada *et al.*

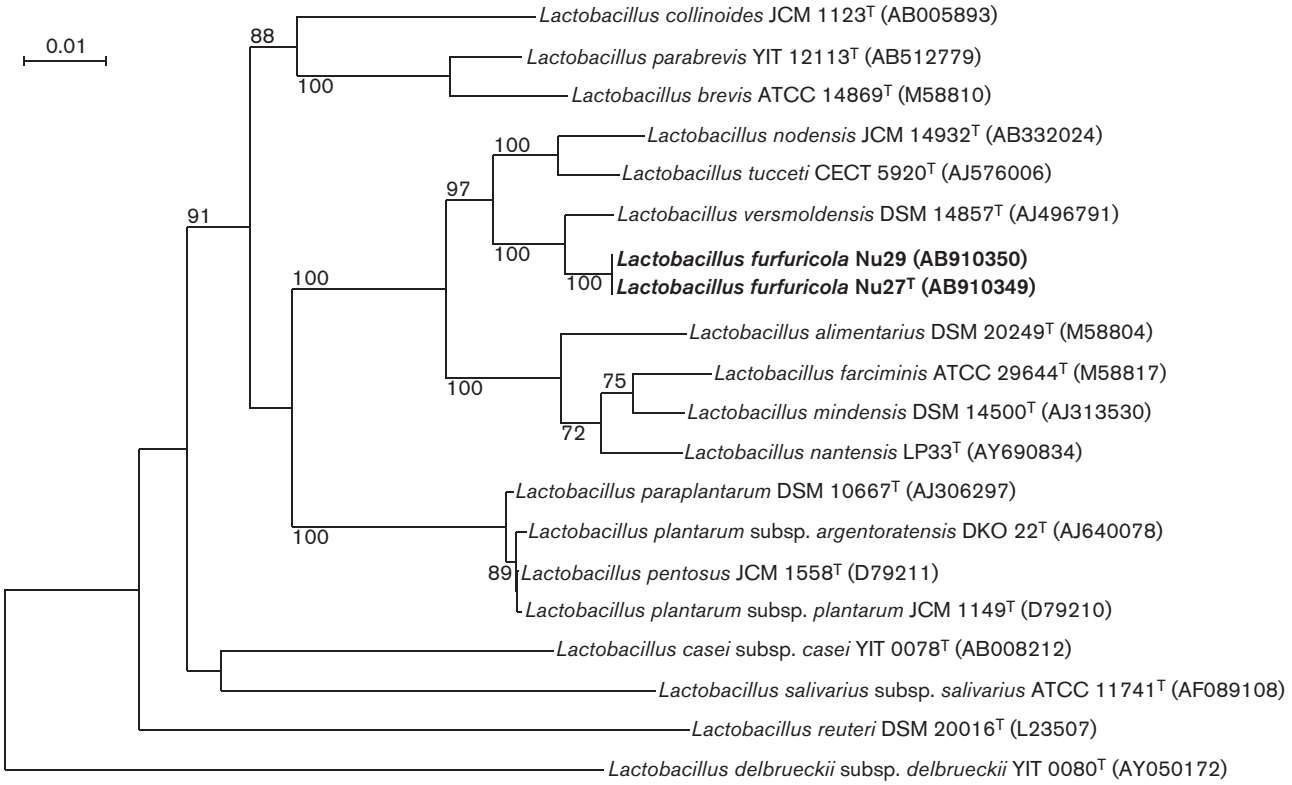

Fig. 1. Phylogenetic relationship of *Lactobacillus furfuricola* sp. nov. and closely related species based on 16S rRNA gene sequences. The tree was reconstructed by the neighbour-joining method. *Lactobacillus delbrueckii* subsp. *delbrueckii* YIT 0080^T was used as an outgroup. Bootstrap percentages >70 % are given at branching points. Bar, 1 % sequence divergence.

(1991). The isomer of lactic acid was analysed by HPLC as described by Manome *et al.* (1998). Acid formation from carbohydrates was assessed by the use of API 50CHL (bioMérieux) in duplicate as recommended by the manufacturer. Preparation of cell wall and determination of the types of peptidoglycan were carried out using methods described by Komagata & Suzuki (1987). Fatty acid methyl esters were prepared from cells grown in MRS agar, incubated for 48 h at 30 °C. The processes including methylation, extraction and the other conditions were as previously described (Sakamoto & Ohkuma, 2013). Cellular fatty acid profiles were determined by following version 2.99B of the Sherlock Microbial Identification System (MIDI) and using version 3.80 of the BHIBLA database.

The characteristics of the novel isolates are given in Table 1 and the species description. All present data were obtained in this study. The isolates did not grow at 15 °C or in the presence of 10 % NaCl in the broth. The isolates produced D- and L-lactic acid from D-glucose. Furthermore, some different characteristics between the isolates and closely related species were found in this study. The major cellular fatty acids in the two novel strains were $C_{16:0}$, $C_{18:1}\omega 9c$ and C_{19} cyc 9,10/:1 (Table S1); these were similar to those recorded for the phylogenetic relatives.

On the basis of results shown in this study, the two isolates are distinct from recognized species. Therefore, the two isolates represent a novel species of the genus *Lactobacillus* for which the name *Lactobacillus furfuricola* sp. nov. is proposed.

Description of *Lactobacillus furfuricola* sp. nov.

Lactobacillus furfuricola [fur.fur.i′co.la. L. n. *furfur* rice bran; L. suff. *-cola* (from L. n. *incola*) dweller, inhabitant; N.L. n. *furfuricola* rice bran-dweller].

Cells are Gram-stain-positive rods measuring 0.8 × 2.5–5.0 μm. Cells occur singly or in pairs, are non-motile, facultatively anaerobic and catalase-negative. Colonies on MRS agar incubated at 30 °C for 2 or 3 days are off-white, smooth, and up to 1.0 mm in diameter. They are homofermentative; no gas is produced from glucose. The main end products from glucose fermentation are D- and L-lactic acid produced at the ratio of 27–30 : 70–73. Nitrate is not reduced. Acid is produced from D-ribose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, maltose, D-galactose (delayed reaction) and aesculin (delayed reaction). The type strain produces acid weakly from salicin and turanose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol,

Table 1. Differential characteristics between *Lactobacillus furfuricola* sp. nov. and phylogenetically related species

Strains: 1, *Lactobacillus furfuricola* sp. nov. (Nu 27^T and Nu 29); 2, *Lactobacillus nodensis* JCM 14932^T; 3, *Lactobacillus tucceti* JCM 18037^T; 4, *Lactobacillus versmoldensis* JCM 16174^T. All data were obtained in the present study except where otherwise indicated. +, Positive; −, negative; W, weakly positive; d, delayed reaction (reaction recorded after 3 days).

Characteristic	1	2	3	4
Growth at/with:				
15 °C	−	+W	+ (−[b])	+
10 % NaCl	−	− (+*[a])	−	+
Acid production from:				
D-Arabinose	−	+	−	−
Ribose	+	+	+	d
Galactose	d	+	−	+
Rhamnose	−	−	+	−
Mannitol	−	−	d	−
Methyl α-D-glucoside	−	−	−	d
Aesculin	d	d	−	d
Salicin	W/−	−	−	W
Maltose	+	−	+	+
Lactose	−	−	−	d
Melibiose	−	−	−	+
Turanose	W/−	−	−	−
L-Fucose	−	−	+	−
Isomer of lactic acid	DL	L (DL[a])	DL	L
Ratio of D-lactic acid : L-lactic acid	30 : 70	6 : 94	33 : 67	7 : 93
DNA G+C content (mol%)	40.0/40.8	38.8	36.3	39.0

*Data from the original description: *a*, Kashiwagi *et al.* (2009); *b*, Chenoll *et al.* (2006).

methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, cellobiose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Growth occurs at 20–35 °C, but not 15 or 40 °C. Growth occurs at pH 4.0–8.5 but not at pH 3.5 or pH 9.0 in MRS broth. Growth is observed in MRS broth containing 7.5 % (w/v) NaCl. The cell-wall peptidoglycan is A4α type (Lys–Asp). The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega 9c$ and C_{19} cyc 9,10/:1.The DNA G+C content ranges from 40.0 to 40.8 mol%.

The type strain is Nu 27^T (=JCM 18764^T=NRIC 0900^T=DSM 27174^T). The DNA G+C content of the type strain is 40.0 mol%. An additional strain of the species is Nu29 (=JCM 18765=NRIC 0901=DSM 27175) and is included in the description. Both strains were isolated from fermented rice bran paste, Nukadoko, in Kanagawa prefecture, Japan.

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