Lactobacillus ozensis sp. nov., isolated from mountain flowers

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Five strains (Mizu2-1^T, Gon2-7, Koba6-1, Koyu2-2 and Miya2-2) of lactic acid bacteria (LAB) were isolated from flowers in Oze National Park, Japan, using anaerobic cultivation. The five isolates were found to share identical 16S rRNA gene sequences. The isolates exhibited low levels of 16S rRNA gene sequence similarity to known LAB; the closest recognized relatives of strain Mizu2-1^T were the type strains of *Lactobacillus kunkeei* (94.9%), *Lactobacillus kefir* (94.1%) and *Lactobacillus buchneri* (93.9%). Comparative analyses of *rpoA* and *pheS* gene sequences demonstrated that the novel isolates did not show significant relationships to other *Lactobacillus* species. The strains were Gram-stain-positive, catalase-negative and heterofermentative. Anaerobic growth was better than aerobic growth. The isolates utilized a narrow range of carbohydrates as sources of carbon and energy, including glucose and fructose. On the basis of phenotypic characteristics and phylogenetic data, the isolates represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus ozensis* sp. nov. is proposed. The type strain is Mizu2-1^T (=JCM 17196^T =DSM 23829^T).

During our studies on the distribution of anaerobes in flowers, we have isolated several species of lactic acid bacteria (LAB) from mountain flowers in Japan. We recently reported the isolation of a novel LAB, *Lactobacillus floricola*, from a mountainous area (Kawasaki *et al.*, 2011). *L. floricola* showed quite low 16S rRNA gene sequence similarity to known LAB, and also showed a narrow range of carbohydrate utilization, using only glucose and fructose. Here, we report the isolation of further isolates that exhibit low 16S rRNA gene sequence similarity to known LAB and show a narrow range of carbohydrate utilization. During the course of our investigations of anaerobes in flowers from several mountainous areas in Japan since 2006, the novel species described in this study has been isolated only from the area of Oze National Park.

Flowers were collected from Oze National Park in 2008–2009. Flower samples were collected using autoclaved

forceps and transferred immediately to sterile tubes. Bacteria were cultivated on MRS agar (Difco) containing 5.0 g calcium carbonate and 15 g agar l⁻¹ at 20–30 °C under anaerobic conditions. After isolation, strains were maintained in MRS broth. The origins of the isolates are shown in Supplementary Fig. S1. The proposed type strain Mizu2-1^T was isolated from a flower of *Inula ciliaris* var. *glandulosa*, a chrysanthemum (Japanese common name, oze-mizugiku), that was collected in August 2008. A large number of colonies (10⁴–10⁸ colonies per flower) were obtained, and the 16S rRNA gene sequences of randomly selected colonies suggested that these isolates represent the most abundant species in each flower (data not shown).

Morphological, physiological and biochemical characteristics were determined according to Okada *et al.* (1992), Holdeman *et al.* (1977) and Gerhardt *et al.* (1981), as described previously (Kawasaki *et al.*, 2011). *Lactobacillus buchneri* NRIC 1040^T, *L. kefir* NRIC 1693^T, *L. kunkeei* DSM 12361^T, *L. floricola* NRIC 0774^T and *L. fructivorans* NRIC 0224^T were used as reference strains in this study. Carbohydrate fermentation tests were conducted in modified MRS broth containing 0.5% (w/v) of various carbohydrates, 1% proteose peptone, 0.1% beef extract, 0.5% yeast extract, 0.02% MgCl₂, 0.2% K₂HPO₄ and 0.005% MnSO₄. Acid production from carbohydrates was also tested by using the API 50CHL system (bioMérieux) in

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Mizu2-1^T, Koba6-1, Koyu2-2, Miya2-2 and Gon2-7 are AB572588–AB572592, respectively. Those for the partial *rpoA* and *pheS* gene sequences of strain Mizu2-1^T are AB574477 and AB574478.

Five supplementary figures are available with the online version of this paper.

triplicate according to the manufacturer's instructions. DNA G+C content determination was carried out according to Mesbah *et al.* (1989). Sequences of the 16S rRNA genes of the isolates were determined using primers 27F (5'-GAGTTTGATCCTGGCTCAG-3'; *Escherichia coli* positions 8–27) and 1525R (5'-AGAAAGGAGGTGATCCAGCC-3'; positions 1525–1545) (Lane *et al.*, 1985). The *rpoA* and *pheS* gene sequences for strain Mizu2-1^T were amplified by PCR with primers *rpoA*-21F (5'-ATGATYGARTTTGAAAAAC-C-3') and *rpoA*-23R (5'-ACHGTRTTTRATDCCDGCRCG-3') and *pheS*-21F (5'-CAYCCNGCHCGYGAYATGC-3') and *pheS*-23-R (5'-GGRTGRACCATVCCNGCHCC-3'), respectively (Naser *et al.*, 2005; Chao *et al.*, 2010).

The closest relatives of the isolates were determined by performing a database search and the sequences of the most closely related strains were retrieved from the NCBI database. Multiple alignments of the sequences were carried out using the program CLUSTAL_X, version 2.0 (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated using the two-parameter method of Kimura (1980). The neighbour-joining method (Saitou & Nei, 1987) was used to construct a phylogenetic tree. The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Phylogenetic trees were also constructed using the maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods by using PHYLIP version 3.65 (Felsenstein, 2005). The 16S rRNA gene sequences of the isolates were compared, and the sequence of Mizu2-1^T was used to search for sequence similarities within the

database. Approximately 1500 bp of the 16S rRNA gene sequences (approx. 400 bp for the *rpoA* gene and 350 bp for the *pheS* gene) of the isolates were used to construct phylogenetic trees.

The 16S rRNA gene sequence of Mizu2-1^T showed low sequence similarity to known species of LAB: all similarities were less than 95 %, and the closest known relatives were the type strains of *Lactobacillus kunkeei* (94.9 %), *L. kefir* (94.1 %) and *L. buchneri* (93.9 %). The isolates clustered most closely with *L. kunkeei* (Edwards *et al.*, 1998) using the neighbour-joining (Fig. 1), maximum-parsimony (Supplementary Fig. S2) and maximum-likelihood (Supplementary Fig. S3) methods. These sequence similarities are significantly lower than those recommended for species differentiation (97 %; Stackebrandt & Goebel, 1994). Therefore, DNA–DNA hybridization between the isolates and known LAB was not carried out. Similarities among the *rpoA* and *pheS* gene sequences of strain Mizu2-1^T and the closest neighbouring species ranged from 78 to 85 % and 78 to 82 %, respectively. On the basis of neighbour-joining analysis of *rpoA* and *pheS* gene sequences, the novel strain did not belong to any known species (Supplementary Figs S4 and S5). Similar topologies were obtained by the minimum-evolution and maximum-parsimony methods (not shown). The DNA G+C content of strain Mizu2-1^T was 41 mol%.

The 16S rRNA gene sequence of strain Mizu2-1^T exhibited 100 % identity to those of the other isolates. To differentiate them, isolates Mizu2-1^T, Gon2-7, Koba6-1, Koyu2-2 and Miya2-2 were analysed by randomly amplified polymorphic DNA-PCR (RAPD-PCR) according to the method of

Fig. 1. Phylogenetic tree showing relationships between strain Mizu2-1^T (*Lactobacillus ozensis* sp. nov.) and related species. The tree was constructed using the neighbour-joining method based on 16S rRNA gene sequences. Bootstrap values above 50 % are given at branching points. *Lactobacillus delbrueckii* BCRC 12195^T was used as an outgroup. Bar, evolutionary distance (K_{nuc}) of 0.02.

Fig. 2. RAPD-PCR fingerprints of strains of *L. ozensis* sp. nov. Lanes: 1, Mizu2-1^T; 2, Gon2-7; 3, Koba6-1; 4, Koyu2-2; 5, Miya2-2; M, size marker (1 kb ladder; GENECAFT). Primer-1 and primer-2 were used; see text for details.

Akopyanz *et al.* (1992) using two primers (primer-1, 5'-GAGGACAAAG; primer-2, 5'-GGCATCGGTT) (Morotomi *et al.*, 2002). RAPD-PCR demonstrated genotypic differences (Fig. 2).

Morphological, physiological and biochemical characteristics of the isolates were determined using MRS broth as the basal medium as described previously (Kawasaki *et al.*,

2011). The detailed characteristics of the isolates are described in the species description. The biochemical characteristics were compared with those of the type strains of the phylogenetic relatives *L. kunkeei*, *L. kefir*, *L. floricola* and *L. fructivorans* (Table 1). The isolates were heterofermentative LAB and produced D- and L-lactic acid from D-glucose, as determined by using L- and D-lactate dehydrogenases (Sigma) (Latorre-Guzman *et al.*, 1977). This finding was confirmed by performing HPLC analysis with a separation column for optical isomers (CRS10W column; Mitsubishi Chemical) (Otsuka *et al.*, 1994; Manome *et al.*, 1998); D- and L-lactic acid were produced at a ratio of 1 : 2. Production of ethanol from glucose was detected by using GC. The strains grew well at 20 and 30 °C (optimum) and grew slowly at 15 and 37 °C. The strains produced acid from a narrow range of carbohydrates, including glucose and fructose, and produced acid weakly from maltose, sucrose and mannitol.

On the basis of the phenotypic characteristics and phylogenetic data, the isolates represent a novel species, for which the name *Lactobacillus ozensis* sp. nov. is proposed.

Description of *Lactobacillus ozensis* sp. nov.

Lactobacillus ozensis (o.zen'sis. N.L. masc. adj. *ozensis* of Oze National Park, Japan, from where the first strains were isolated).

Cells are Gram-stain-positive, non-spore-forming, non-motile rods, 0.5 × 3–4 µm, and occur singly, in pairs or in

Table 1. Differential characteristics of strain Mizu2-1^T (*Lactobacillus ozensis* sp. nov.) and closely related lactobacilli

Strains: 1, Mizu2-1^T; 2, *L. floricola* NRIC 0774^T; 3, *L. kunkeei* DSM 12361^T; 4, *L. kefir* NRIC 1693^T; 5, *L. fructivorans* NRIC 0224^T. Data were obtained in this study. +, Positive; –, negative; w, weakly positive. All strains fermented fructose.

Characteristic	1	2	3	4	5
Lactic acid isomer(s)	DL	L	L	DL	DL
Type of fermentation*	Hetero	Homo	Hetero	Hetero	Hetero
Fermentation of:†					
Arabinose	–	–	–	+	+
Cellobiose	–	–	–	–	+
Galactose	–	–	–	+	+
Lactose	–	–	–	+	+
Maltose	w	–	–	+	+
Mannitol	w	–	w	w	+
Mannose	–	–	–	–	+
Melezitose	–	–	–	–	+
Raffinose	–	–	–	–	+
Ribose	–	–	–	w	+
Salicin	–	–	–	–	+
Sorbitol	–	–	–	–	+
Sucrose	w	–	+	w	+
Trehalose	–	–	–	w	+

*Homo, Homofermentative; Hetero, heterofermentative.

†Carbohydrate fermentation tests were confirmed by using API 50CHL. Mizu2-1^T produced acid from D-glucose and D-fructose of the 48 carbohydrates in API 50CHL.

short chains. Catalase-negative. Colonies develop well on MRS agar plates under anaerobic conditions, and growth is inhibited under aerobic conditions. Colonies on MRS agar are white, smooth and approximately 2 mm in diameter after incubation for 2 days at 30 °C under anaerobic conditions. Heterofermentative. Gas is produced from glucose. Both D- and L-lactic acid are produced as end products from D-glucose. Nitrate is not reduced. Acid is produced from D-glucose and D-fructose, and is produced weakly from maltose, sucrose and D-mannitol; no acid production from D-galactose, D-mannose, D-arabinose, D-xylose, melibiose, trehalose, melezitose, D-sorbitol, D-ribose, cellobiose, lactose, raffinose, D-gluconate, L-rhamnose or salicin. Grows at 20–30 °C and grows slowly at 15 and 37 °C. Grows at 30 °C in the presence of 1.0 % (w/v) NaCl but not 1.5 % (w/v) NaCl. Cells do not contain *meso*-diaminopimelic acid in their peptidoglycan. The DNA G + C content of the type strain is 41 mol%.

The type strain is Mizu2-1^T (=JCM 17196^T =DSM 23829^T), isolated from a flower of *Inula ciliaris* var. *glandulosa*, a chrysanthemum (Japanese common name oze-mizugiku), that was collected from Oze National Park in August 2008. Four additional strains, Gon2-7 (=JCM 17197), Koba6-1 (=JCM 17198), Koyu2-2 (=JCM 17199) and Miya2-7 (=JCM 17200), are included in this species (details of isolation in legend to Supplementary Fig. S1). All five strains were isolated from mountain flowers in the area of Oze National Park.

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