Lactobacillus floricola sp. nov., lactic acid bacteria isolated from mountain flowers

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Five strains (Ryu1-2^T, Gon2-9, Ryu4-3, Nog8-1 and Aza1-1) of lactic acid bacteria were isolated from flowers in mountainous areas in Japan, Oze National Park, Iizuna mountain and the Nikko area. The five isolates were found to share almost identical (99.6–100 % similar) 16S rRNA gene sequences and were therefore deemed to belong to the same species. These isolates exhibited low levels of 16S rRNA gene sequence similarity to known lactic acid bacteria; the closest recognized relatives to strain Ryu1-2^T were the type strains of *Lactobacillus hilgardii* (92.8 % similarity), *Lactobacillus kefiri* (92.7 %), *Lactobacillus composti* (92.6 %) and *Lactobacillus buchneri* (92.4 %). 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 homofermentative. 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, these isolates represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus floricola* sp. nov. is proposed. The type strain is Ryu1-2^T (=NRIC 0774^T =JCM 16512^T =DSM 23037^T).

Lactobacillus strains have been isolated from several plant sources such as fruit, grass, leaves, tree sap, flowers, fermented vegetables and fermented beverages such as wine, malt whisky, shochu and beer (summarized by Hammes & Hertel, 2009; Orla-Jensen, 1919; Douglas & Cruess, 1936; Keddie, 1959; Carr & Davies, 1970; Wibowo *et al.*, 1985; Edwards *et al.*, 1998; Bohak *et al.*, 1998; Simpson *et al.*, 2001; Endo & Okada, 2007; Endo *et al.*, 2009; Michaylova *et al.*, 2007; Irisawa & Okada, 2009). In the 1960s, Mundt and colleagues reported the distribution of lactic acid bacteria (LAB) in flowers found in a national park in the United States (Mundt, 1963; Mundt *et al.*, 1967). During our studies on the distribution of anaerobes in flowers, we have isolated strains of a novel *Lactobacillus* species from several flower samples found in mountainous areas of national parks in Japan.

Flowers were collected from mountainous areas (over 1000 m elevation) in Japan in the years 2006–2009. We also collected flowers from Oze National Park in the years 2008–2009. Flower samples were collected using autoclaved forceps, and transferred immediately to sterile tubes. Bacteria were cultivated at 20–30 °C under anaerobic conditions on MRS agar (Difco), containing 5.0 g calcium carbonate and 15 g agar l^{-1}. After isolation, the strains were maintained in MRS broth. We isolated five strains (Ryu1-2^T, Gon2-9, Ryu4-3, Nog8-1 and Aza1-1) from different flower sources found in mountainous locations in Japan. The origins of the isolates are shown in Supplementary Fig. S1, available in IJSEM Online. A large

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Ryu1-2^T, Gon2-9, Ryu4-3, Nog8-1 and Aza1-1 are AB523780–AB523784, respectively; those for the partial *rpoA* gene sequences of Ryu1-2^T and *L. composti* DSM 18527^T are AB568092 and AB568093, and those for the partial *pheS* gene sequences of Ryu1-2^T and *L. composti* DSM 18527^T are AB568094 and AB568095.

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

number of colonies (10^4–10^8 colonies per single 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 (unpublished data).

Morphological, physiological and biochemical characteristics were determined according to the methods of Okada *et al.* (1992), Holdeman *et al.* (1977) and Gerhardt *et al.* (1981). *Lactobacillus buchneri* NRIC 1040^T, *L. composti* DSM 18527^T, *L. hilgardii* NRIC 1060^T, *L. kefiri* NRIC 1693^T and *L. salivarius* NRIC 0739^T were used as experimental reference strains in this study. Carbohydrate fermentation tests were conducted in modified MRS broth containing 0.5 % (w/v) of various carbohydrates. Acid production from carbohydrates was also tested by using the API 50CHL system (bioMérieux) in triplicate according to the manufacturer's instructions. DNA G+C contents were determined according to Mesbah *et al.* (1989). Sequences of the 16S rRNA genes of the isolates were determined using the primers 27F (5′-GAGTTTGATC-CTGGCTCAG-3′; *Escherichia coli* positions 8–27) and 1525R (5′-AGAAAGGAGGTGATCCAGCC-3′; *E. coli* positions 1525–1545) (Lane *et al.*, 1985). The *rpoA* and *pheS* gene sequences for strain Ryu1-2^T and *L. composti* DSM 18527^T were amplified by PCR with degenerate primers rpoA-21F (5′-ATGATYGARTTTGAAAAACC-3′) and rpoA-23R (5′-ACHGTRTTRATDCCDGCRCG-3′) and pheS-21F (5′-CAYCCNGCHCGYGAYATGC-3′) and pheS-23-R (5′-GG-RTGRACCATVCCNGCHCC-3′), respectively (Naser *et al.*, 2005; Chao *et al.*, 2010).

The closest relatives of the isolates were determined by performing a search against public databases, and the sequences of the most closely related species 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 Ryu1-2^T was used to search for sequence similarities within the database. Sequences of approx. 1500 bp of the 16S rRNA gene (approx. 400 bp for *rpoA* and 350 bp for *pheS*) were used to construct phylogenetic trees. The sequence of Ryu1-2^T showed quite low sequence similarity to known species of LAB: all similarities were less than 93 %, and the closest known relatives were the type strains of *L. hilgardii* (92.8 %), *L. kefiri* (92.7 %), *L. composti* (92.6 %) and *L. buchneri* (92.4 %). The isolates clustered most closely with *L. composti* (Endo & Okada, 2007) using the neighbour-joining and maximum-parsimony methods (Fig. 1 and Supplementary Fig. S2a) and with *L. salivarius* (Rogosa *et al.*, 1953) using the maximum-likelihood method (Supplementary Fig. S2b). These sequence similarities are significantly lower than those recommended for species differentiation (99 %; Stackebrandt & Ebers, 2006). Therefore, DNA–DNA hybridization between the isolates and known LAB was not carried out. The similarities among the *rpoA* and *pheS* gene sequences of the proposed type strain and the closest neighbouring species were 65–70 and 57–71 %, respectively. On the basis of neighbour-joining analysis of the *rpoA* and *pheS* gene sequences (Supplementary Fig. S3), the novel strain did not belong to any known species. Similar topologies were obtained by the minimum evolution and maximum-parsimony methods (not shown). The DNA G+C content of the strain Ryu1-2^T was 48 mol%.

The 16S rRNA gene sequence of strain Ryu1-2^T was 100 % identical to that of strain Gon2-9 and exhibited high sequence identity to those of strains Ryu4-3 (one base difference), Nog8-1 (99.6 %) and Aza1-1 (99.6 %). The sequences of Nog8-1 and Aza1-1 were identical. To attempt to differentiate these strains, strains Ryu1-2^T, Gon2-9, Ryu4-3, Nog8-1 and Aza1-1 were analysed by randomly amplified polymorphic DNA (RAPD)-PCR according to the method of Akopyanz *et al.* (1992) using two primers (primer-1, 5′-GAGGACAAAG; primer-2, 5′-GGCATCG-GTT) (Morotomi *et al.*, 2002). RAPD-PCR demonstrated genotypic differences between the strains (Fig. 2). We

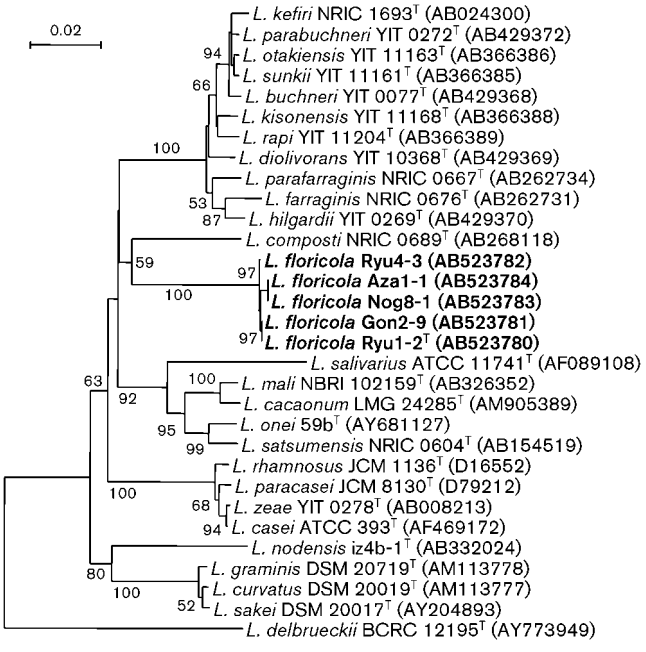

Fig. 1. Phylogenetic tree showing the relationship between the novel strains from mountain flowers and related species. The tree was constructed using the neighbour-joining method based on 16S rRNA gene sequences. Bootstrap values above 50 % are given at branching points. Bar, evolutionary distance (K_{nuc}) of 0.02.

concluded that the isolated strains are widely distributed in mountain flowers but are not specific to a particular area.

Morphological, physiological and biochemical characteristics of the isolates were determined using MRS broth as a basal medium. Detailed characteristics are given in the species description. The biochemical characteristics were compared with those of the phylogenetic relatives *L. hilgardii*, *L. composti*, *L. kefiri*, *L. salivarius* and *L. buchneri* (Table 1). The isolates were homofermentative LAB and produced L-lactic acid from D-glucose, as determined by using L- and D-lactate dehydrogenase (Sigma) (Latorre-Guzman *et al.*, 1977). This finding was also confirmed by performing HPLC analysis with a separation column for optical isomers (CRS10W column; Mitsubishi Chemical) (Otsuka *et al.*, 1994; Manome *et al.*, 1998). Production of lactic acid but not ethanol from glucose was detected by using gas chromatography. The strain grew well at 20 and 30 °C (optimum), slowly at 15 °C and not at all at 10 or 37 °C. The strain produced acid from a narrow range of carbohydrates such as glucose and fructose. Growth by utilization of glucose was relatively better than that with fructose, as determined by measuring maximum growth by monitoring the OD_{660} for 24–48 h at 30 °C.

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

Description of *Lactobacillus floricola* sp. nov.

Lactobacillus floricola (flo.ri′co.la. L. n. *flos -oris* a flower; L. suff. *-cola* derived from L. n. *incola* a dweller; N.L. n. *floricola* flower-dweller).

Cells are Gram-stain-positive at the early stages of growth but are not clearly stained at the late exponential to stationary phase. They are non-spore-forming, non-motile rods, 0.5 × 2–4 μm, and occur singly, in pairs or in short chains. Catalase-negative. Colonies develop well on MRS agar plates under both anaerobic and aerobic (air) conditions. Colonies on MRS agar are yellowish-white, smooth and approx. 1–2 mm in diameter after incubation for 2 days at 30 °C. Homofermentative. No gas is produced from glucose. L-Lactic acid is produced as the end product from glucose. Nitrate is not reduced. Acid is produced from D-glucose and D-fructose; weak production is observed from starch. No acid is produced from D-galactose, D-mannose, D-arabinose, D-xylose, maltose, melibiose, sucrose, trehalose, melezitose, D-mannitol, D-sorbitol, D-ribose, cellobiose, lactose, raffinose, D-gluconate, L-rhamnose or salicin. Utilization of glucose is relatively better than that of fructose. Cells grow at 20–30 °C and grow slowly at 15 °C, but not at 10 or 37 °C. Cells grow at 30 °C in the presence of 5.5 % (w/v) NaCl but not 6.5 % (w/v). Cells do not contain *meso*-diaminopimelic acid in their peptidoglycan. The DNA G+C content of the type strain is 48 mol%.

The type strain is Ryu1-2^T (=NRIC 0774^T =JCM 16512^T =DSM 23037^T), isolated from a flower of *Caltha palustris* (Japanese common name ryukinka) in the Oze National Park in June 2008. Four additional strains, Aza1-1 (=NRIC 0775 =JCM 16513), Nog8-1 (=NRIC 0776 =JCM 16514), Gon2-9 (=NRIC 0777 =JCM 16515) and Ryu4-3 (=NRIC 0778 =JCM 16516) are included in this species (details in Supplementary Fig. S1).

Table 1. Differential characteristics of strain Ryu1-2^T and closely related lactobacilli

Strains: 1, Ryu1-2^T; 2, *L. composti* DSM 18527^T; 3, *L. hilgardii* NRIC 1060^T; 4, *L. kefiri* NRIC 1693^T; 5, *L. salivarius* NRIC 0739^T; 6, *L. buchneri* NRIC 1040^T. Data were obtained in this study. +, Positive; W, weakly positive; −, negative. Carbohydrate fermentation tests were confirmed by using API 50CHL; strain Ryu 1-2^T produced acid from D-glucose and D-fructose of the 49 carbohydrates in API 50CHL. None of the strains fermented cellobiose, and all strains fermented fructose.

Characteristic	1	2	3	4	5	6
Lactic acid isomer(s)	L	DL	DL	DL	L	DL
Fermentative behaviour*	Ho	He	He	He	Ho	He
Fermentation of:						
Arabinose	−	+	−	+	−	+
Galactose	−	+	−	+	+	+
Lactose	−	−	−	+	+	−
Maltose	−	+	+	+	+	+
Mannitol	−	+	−	−	+	−
Mannose	−	+	−	−	+	−
Melezitose	−	+	−	−	−	+
Rhamnose	−	W	−	−	W	−
Ribose	−	−	+	W	−	+
Salicin	−	W	−	−	−	−
Sorbitol	−	+	−	−	+	−
Trehalose	−	+	−	W	+	−

*Ho, Homofermentative; He, heterofermentative.

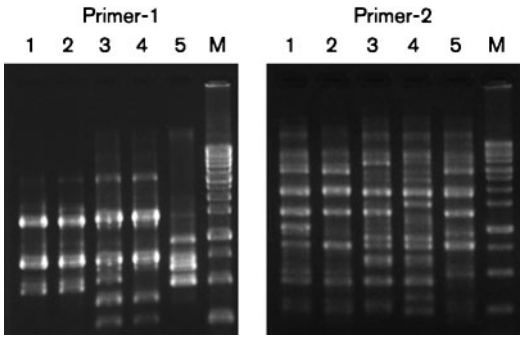

Fig. 2. RAPD-PCR fingerprints of strains of *Lactobacillus floricola* sp. nov. Lanes: 1, Ryu1-2^T; 2, Ryu4-3; 3, Nog8-1; 4, Aza1-1; 5, Gon2-9; M, size marker (1 kb ladder; GENECRAFT). Primer-1 and primer-2 were used (see text).

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