Lactobacillus florum sp. nov., a fructophilic species isolated from flowers

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Three strains of fructophilic lactic acid bacteria were isolated from flowers in South Africa. The isolates formed a subcluster in the *Lactobacillus buchneri* phylogenetic group, closely related to *Lactobacillus fructivorans*, *Lactobacillus homohiochii*, *Lactobacillus lindneri* and *Lactobacillus sanfranciscensis* according to phylogenetic analysis based on the 16S rRNA gene sequences. Levels of DNA–DNA relatedness indicated that the three strains belonged to the same taxon and formed a genetically distinct group, well separated from their phylogenetic relatives. The three strains produced acids from only two of the 49 carbohydrates tested, i.e. D-glucose and D-fructose. D-Fructose was more rapidly fermented than D-glucose. Good growth was recorded on D-fructose or D-glucose in the presence of external electron acceptors. However, delayed growth was recorded on D-glucose without electron acceptors. The novel strains produced lactic acid, ethanol and acetic acid from D-glucose at a ratio of 1 : 0.8 : 0.2. These characteristics were distinct from other species of the genus *Lactobacillus*. Based on the data provided, the three isolates represent a fructophilic and novel species of the genus *Lactobacillus*, for which the name *Lactobacillus florum* sp. nov. is proposed. The type strain is F9-1^T (=JCM 16035^T=DSM 22689^T=NRIC 0771^T).

Fructophilic lactic acid bacteria (LAB) are a group of LAB described recently (Endo & Okada, 2008). These organisms are known to have unique characteristics, i.e. they grow well on D-fructose or D-glucose in the presence of external electron acceptors, but grow poorly on D-glucose without electron acceptors (Endo & Okada, 2008). Pyruvate and oxygen are used as electron acceptors. At the time of writing, only five species with these characteristics have been described, *Fructobacillus fructosus*, *Fructobacillus durionis*, *Fructobacillus ficulneus* and *Fructobacillus pseudo-ficulneus* (Endo & Okada, 2008) and *Lactobacillus kunkeei* (Endo *et al.*, 2009).

During a study of fructophilic LAB, three strains were isolated from flowers in South Africa (Endo *et al.*, 2009). The isolates shared several common characteristics with known fructophilic LAB, but also had a few different characteristics. They formed a subcluster within the *Lactobacillus buchneri*

cluster on phylogenetic analysis based on their 16S rRNA gene sequences and were closely related to *Lactobacillus lindneri*. Levels of DNA–DNA similarity and biochemical characteristics showed clear separation of the isolates from *L. lindneri*. This paper describes the taxonomic study of the three novel isolates and their classification as a novel species of the genus *Lactobacillus*.

The three novel strains were isolated from flowers of peony (*Paeonia suffruticosa*) and bietou (*Chrysanthemoides monilifera*) in the Stellenbosch and Hermanus area, Western Cape, South Africa, between September and October 2008. Fresh flowers were collected in sterile plastic bags and crushed. A 1–5 ml aliquot of FYP broth (1⁻¹: 10 g D-fructose, 10 g yeast extract, 5 g polypeptone, 2 g sodium acetate, 0.5 g Tween 80, 0.2 g MgSO₄·7H₂O, 0.01 g MnSO₄·4H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g NaCl, 0.05 g cycloheximide, 0.05 g sodium azide; pH 6.8), was added to the crushed samples and the bags were incubated at 30 °C for 24 h. After incubation, a loopful of each sample was inoculated into 30 % FYP broth (FYP broth supplemented with 300 g l⁻¹ D-fructose) and incubated aerobically on an orbital shaker (120 r.p.m.) at 30 °C until visible growth was observed. The 30 % FYP broth was used for selection of fructophilic LAB. Species of the genus *Fructobacillus* are known to resist high osmotic pressure (Endo & Okada,

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Lactobacillus florum* strains F9-1, F9-2 and F17 are AB498045, AB498046 and AB498047, respectively.

Additional phylogenetic trees based on maximum-likelihood and maximum-parsimony analysis of the 16S rRNA gene sequences of the novel strains are available with the online version of this paper.

2008) and *F. ficulneus* was originally isolated using broth containing 30 % glucose (Antunes *et al.*, 2002). After visible growth was observed, cultures from 30 % FYP broth were streaked onto FYP agar containing (1^{-1}) 5 g CaCO_3 and 12 g agar. Plates were incubated at 30 °C under aerobic conditions until colonies were visible. Colonies were selected based on morphological differences (colony size and shape, and clearance zone formed from hydrolysis of the CaCO_3 by lactic acid), inoculated into FYP broth and GYP broth and incubated statically for 24 h at 30 °C. GYP broth differed from FYP broth by containing 10 g l^{-1} D-glucose instead of D-fructose.

The three novel strains, F9-1^T, F9-2 and F17, grew well in FYP broth, but poorly in GYP broth and were thus regarded as fructophilic LAB. They were preserved at –80 °C in nutrient broth containing 20 % (v/v) glycerol.

The 16S rRNA gene sequences of the three isolates were determined according to a previously described method (Endo & Okada, 2005). The closest recognized relatives of the isolates were determined by performing database searches and sequences of closely related species were retrieved from the DDBJ. Multiple alignments of the sequences were carried out with the CLUSTAL_X program version 1.18 (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Phylogenetic trees were also constructed by using the maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods with PHYLIP version 3.65 as described previously (Endo & Okada, 2006). The 16S rRNA gene sequences determined for the three isolates were compared with each other and the sequence of strain F9-1^T was used to search for sequence similarity using the DDBJ database. Approximately 1450 bp of the 16S rRNA gene sequences of the novel isolates and related species were used to construct the phylogenetic trees. The three new strains shared 99.9 % sequence similarity based on the 16S rRNA gene. The highest gene sequence similarities to strain F9-1^T were recorded with *L. lindneri* and *L. sanfranciscensis* (95.4 % and 93.7 %, respectively). The three strains produced a subcluster with *L. fructivorans*, *L. homohiochii*, *L. lindneri* and *L. sanfranciscensis* inside the *L. buchneri* phylogenetic group on the basis of neighbour-joining analysis (Fig. 1). Identical tree topologies were obtained by using the maximum-likelihood and maximum-parsimony analysis (see Supplementary Figs S1 and S2 in IJSEM Online).

Levels of DNA–DNA relatedness between the new isolates and *L. lindneri* JCM 11027^T, and the DNA G + C contents of the isolates were determined according to methods described by Kitahara *et al.* (2001). Extraction and purification of bacterial DNA was performed according

to the methods of Kitahara *et al.* (2001) and purified by the method of Saito & Miura (1963). Strains of *L. fructivorans*, *L. homohiochii* and *L. sanfranciscensis* were not used in this study because the sequence similarities between the new isolates and the type strains of these three species were lower than the recommended value for species differentiation as proposed by Stackebrandt & Ebers (2006). The new isolates showed high levels of DNA–DNA relatedness (ranging from 89 to 100 %) among them. Therefore, it was concluded that the new isolates belonged to the same taxon. In contrast, the isolates showed low levels of DNA–DNA relatedness (ranging from 8 to 12 %) to *L. lindneri* JCM 11027^T. The G + C content of the three isolates was 42 mol%.

To differentiate the isolates, randomly amplified polymorphic DNA (RAPD) fingerprinting was performed according to the method of Endo & Okada (2006). Three primers described previously (Endo & Okada, 2006), primer D (5'-GAGGACAAAG), primer E (5'-GGCGTCGGTT) and primer F (5'-GGCCACGGAA), and another three primers, primer OPL-01 (5'-GGCATGACCT), primer OPL-02 (5'-TGGGCGTCAA) and primer OPL-03 (5'-CCAGCAGCTT) (RAPD 10 mer kit, Operon Technologies Inc.) were used for RAPD-PCR. The DNA fingerprints obtained indicated that the three strains were genetically similar, but were different at the strain level (Fig. 2).

Morphological, physiological and biochemical characteristics were determined by using previously described methods (Endo & Okada, 2005), except that API 50 CHL galleries (bioMérieux) were used for the determination of acid production from carbohydrates. FYP broth was used as a basal medium. The detailed characteristics for the novel isolates are given in the species description and are compared with the characteristics of their phylogenetic neighbours *L. fructivorans*, *L. homohiochii*, *L. lindneri* and *L. sanfranciscensis* in Table 1. From the 49 carbohydrates tested, the new isolates produced acids from only D-glucose and D-fructose. To the authors' knowledge, this result represents the lowest number of fermented carbohydrates thus far described for LAB. D-Fructose was fermented within 24 h, but D-glucose was fermented after 48 h. The novel isolates could be distinguished from their phylogenetic relatives by their acid production patterns from carbohydrates (Table 1). The new isolates grew well at acidic to weak alkaline pH (4.0–7.5), whereas their phylogenetic relatives were acidophilic. Growth at pH 6.5–7.5 is thus a key characteristic that differentiated the new isolates from phylogenetically closely related species. Catalase activity was not detected from the three strains grown in FYP broth under aerobic conditions but was detected when the novel strains were cultured on FYP agar containing 5 % (v/v) sheep blood under aerobic conditions. The strains produced DL-lactic acid, ethanol and acetic acid at a ratio of 1:0.8:0.2 (Endo *et al.*, 2009). The three strains reached optical density values (660 nm) of 1.0 after 2 days incubation in the presence of 200 g l^{-1} D-fructose. The novel strains took 3–4 days to reach optical density values of 1.0 in the presence of 300 g l^{-1} D-fructose.

Fig. 1. Phylogenetic relationships of strains F9-1^T, F9-2 and F17 with related species based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method. *Lactococcus lactis* NCDO 604^T was used as an outgroup. Bootstrap percentages >70 % are given at branching points. Bar, 0.01 evolutionary distance (K_{nuc}).

Growth of the three strains was slow on D-glucose and fast on D-fructose (Endo *et al.*, 2009). Growth on D-glucose was enhanced in the presence of 1 % (w/v) pyruvate or under aerobic conditions, suggesting that an electron acceptor enhanced the growth of the novel strains. Growth enhancement by the presence of pyruvate is unknown for the phylogenetic relatives of the novel strains. Aerobic culturing repressed the growth of the three novel strains on

agar plates. Based on this and on the end products from glucose, the new strains are classified as facultatively fructophilic LAB (Endo *et al.*, 2009). The three new isolates represent the only facultatively fructophilic LAB so far described. *F. fructosus*, *F. durionis*, *F. ficulneus*, *F. pseudophiliculus* and *L. kunkee* have been described as 'obligately' fructophilic LAB (Endo & Okada, 2008; Endo *et al.*, 2009). The distinguishing features between the 'obligately' and

Fig. 2. RAPD-PCR fingerprints of the three novel strains. Primers D, E and F (a) and primers OPL-1, OPL-2 and OPL-3 (b) were used. Lanes: M, size marker (1 kb ladder, Fermentas); 1, F9-1^T; 2, F9-2; 3, F17.

Table 1. Characteristics useful for differentiation between the novel strains and closely related species

Strains/taxa: 1, F9-1^T, F9-2 and F17; 2, *L. lindneri* (data from Back *et al.*, 1996); 3, *L. sanfranciscensis* (Kandler & Weiss, 1986; Hammes & Hertel, 2006); 4, *L. fructivorans* (Kandler & Weiss, 1986); 5, *L. homohiochii* (Kandler & Weiss, 1986; Hammes & Hertel, 2006). +, Positive; –, negative; w, weakly positive; D, 11–89 % of strains are positive.

Characteristic	1	2	3	4	5
Acid from					
D-Fructose	+	D	–	+	+
D-Galactose	–	–	+	–	–
Gluconate	w	–	+	–	–
Maltose	–	+	+	D	+
D-Mannose	–	–	–	–	+
D-Ribose	–	–	D	w	D
Sucrose	–	–	D	D	–
DNA G + C content (mol%)	42	35	36–38	38–40	35–38
Growth at pH 7.5	+	–	–	–	–

facultatively fructophilic LAB are growth on D-glucose without electron acceptors and production of ethanol from D-glucose (Endo *et al.*, 2009). ‘Obligately’ fructophilic LAB are unable to grow well on glucose in the absence of electron acceptors and do not produce ethanol from D-glucose (Endo *et al.*, 2009).

The data show that the three new isolates from flowers are phylogenetically and biochemically distinct from recognized species in the genus *Lactobacillus*. Thus, the new isolates represent a novel species for which the name *Lactobacillus florum* sp. nov. is proposed. The type strain is F9-1^T (=JCM 16035^T=DSM 22689^T=NRIC 0771^T).

Description of *Lactobacillus florum* sp. nov.

Lactobacillus florum (flo'rum. L. n. *flos* -oris flower; L. gen. pl. n. *florum* of flowers, from which the type strain was isolated).

Cells are Gram-positive, non-motile rods, measuring 0.8 × 1.5–7 µm. Cells occur singly or in pairs and chains. Facultatively anaerobic. Tests for catalase are usually negative but catalase activity is found in cells grown on FYP agar containing 5 % sheep blood. Colonies on FYP agar are beige, smooth and approximately 1–2 mm in diameter after incubation for 3 days under anaerobic conditions. Surface growth under aerobic conditions is poor. Heterofermentative and produces lactic acid, ethanol and acetic acid from D-glucose at a ratio of 1:0.8:0.2. Gas is produced from D-glucose. D-Lactate and L-lactate are produced at a ratio of 1:1. Nitrate is not reduced. Acid is produced only from D-glucose and D-fructose. D-Fructose is fermented faster than D-glucose. Potassium gluconate is weakly fermented. Acid is not produced from L-arabinose, D-arabitol, N-acetylglucosamine, maltose, ribose, D-arabinose, L-arabitol, adonitol, amygdalin, arbutin, cellobiose,

dulcitol, aesculin, erythritol, D-fucose, L-fucose, β-gentio-biose, 2- and 5-ketogluconate, methyl α-D-glucoside, glycerol, glycogen, inositol, inulin, D-lyxose, D-mannose, methyl α-D-mannoside, melezitose, raffinose, rhamnose, sucrose, salicin, starch, sorbitol, L-sorbose, D-tagatose, trehalose, turanose, xylitol, L-xylose, methyl β-xyloside, D-galactose, lactose, mannitol, melibiose or D-xylose. Dextran is not produced from sucrose. Pyruvate enhances growth of the strains. Strains grow at 15 °C, but not at 45 °C. Cells grow at pH 4.0–8.0 or in the presence of 5 % (w/v) NaCl. Osmotolerant. Cells grow in the presence of 300 g l^{–1} D-fructose. Cells do not contain meso-diaminopimelic acid in their cell-wall peptidoglycan. The DNA G + C content is 42 mol%.

The type strain, F9-1^T (=JCM 16035^T=DSM 22689^T=NRIC 0771^T), was isolated from peony and bietou flowers (Endo *et al.*, 2009) collected at Stellenbosch and Hermanus, Western Cape, South Africa, in 2008.

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