

Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium

Akihito Endo,^{1,2†} Tomohiro Irisawa,^{3†} Yuka Futagawa-Endo,¹ Katsumi Takano,³ Maret du Toit,⁴ Sanae Okada³ and Leon M. T. Dicks¹

Correspondence

Akihito Endo

pegaman@hotmail.co.jp

¹Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

²Functional Foods Forum, University of Turku, Turku 20014, Finland

³Department of Applied Biology and Chemistry, Faculty of Applied Bio-Science, Tokyo University of Agriculture, Tokyo 156-8502, Japan

⁴Institute for Wine Biotechnology, University of Stellenbosch, Stellenbosch 7600, South Africa

Lactobacillus kunkeei is an inhabitant of fructose-rich niches and is a potential member of the fructophilic lactic acid bacteria. In the present study, the phylogenetic and biochemical characteristics of the type strain and eight isolates of *L. kunkeei*, originating from wine, flowers and honey, were studied. The nine isolates, including the type strain, formed a well-defined phylogenetic subcluster based on the analysis of 16S rRNA gene sequences. The subcluster was not closely related to other subclusters in the *Lactobacillus* phylogenetic group. Biochemically, the eight new isolates showed typical fructophilic characteristics. The eight isolates grew poorly on glucose, but grew well on fructose. Good growth on glucose was only recorded in the presence of electron acceptors. The type strain of *L. kunkeei* differed from the other isolates only on the basis of poor growth on fructose. Although they belong to a group of obligately heterofermentative lactic acid bacteria, all nine isolates, including the type strain, produced almost equimolar amounts of lactic acid and acetic acid and very little ethanol from glucose. Eight of the isolates can thus be regarded as typical 'obligately' fructophilic lactic acid bacteria. Although the type strain of *L. kunkeei* was phenotypically slightly different from the other isolates, it possessed several important fructophilic characteristics. On the basis of the evidence gathered in this study, the type strain of *L. kunkeei* is recognized as a member of the 'obligately' fructophilic lactic acid bacteria.

Fructophilic lactic acid bacteria are a group of lactic acid bacteria that prefer fructose as a growth substrate and do not grow well with glucose (Endo *et al.*, 2009). Several interesting characteristics have been reported for these organisms. Fructophilic lactic acid bacteria grow very weakly on glucose as the sole carbon source, but are able to grow very well on glucose in the presence of external electron acceptors such as oxygen, pyruvate and fructose (Endo & Okada, 2008; Endo *et al.*, 2009). Biochemically, they belong to the obligately heterofermentative lactic acid bacteria, but they produce almost equimolar amounts of lactic acid and acetic acid and very little ethanol from

glucose. Only a few carbohydrates are fermented and the species are grouped as either 'obligately' or facultatively fructophilic (Endo *et al.*, 2009). The two groups are differentiated based on their growth on glucose and their ethanol production from glucose. To date, *Fructobacillus durionis*, *F. ficulneus*, *F. fructosus*, *F. pseudoficulneus* and a recently described species, *F. tropaeoli*, have been classified as 'obligately' fructophilic lactic acid bacteria (Endo & Okada, 2008; Endo *et al.*, 2011). *Lactobacillus florum* is facultatively fructophilic (Endo *et al.*, 2010). *Lactobacillus kunkeei*, isolated from flowers, also displayed several fructophilic characteristics and has recently been characterized as an 'obligately' fructophilic lactic acid bacteria (Endo *et al.*, 2009).

L. kunkeei, originally isolated from fermenting wine, was identified as a novel species by phylogenetic analysis based on 16S rRNA gene sequences (Edwards *et al.*, 1998). DNA–DNA hybridization studies and determination of the DNA

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the *L. kunkeei* isolates investigated in this study are listed in Table 1.

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

G+C content of the novel strain were not conducted in the original study. The species was not well characterized biochemically and the fructophilic characteristics of the species were not mentioned (Edwards *et al.*, 1998). Therefore, it is not clear whether fructophilic characteristics are common to all isolates of *L. kunkeei* that inhabit various different niches. In the present study, the genotypic and biochemical characterization of several *L. kunkeei* isolates is discussed.

Eight isolates of *L. kunkeei* derived from wine, flowers and honey in South Africa and Japan were included in the present study (Table 1). The strains used in this study were obtained from the NODAI Culture Collection Center (NRIC), Tokyo University of Agriculture, Japan, and from the Institute for Wine Biotechnology (IWB), University of Stellenbosch, South Africa. *L. kunkeei* DSM 12361^T was used as a reference strain. The isolates were pre-cultured in MRS broth (Biolab Diagnostics) under aerobic conditions by shaking on an orbital shaker (120 r.p.m.) for 24 h at 30 °C, unless otherwise indicated.

The 16S rRNA gene sequences of the eight isolates were determined according to a previously described method (Endo & Okada, 2005). The 16S rRNA gene sequence of the type strain of *L. kunkeei* was retrieved from the NCBI database. The closest recognized relatives of the isolates were determined by performing searches in the NCBI database and the sequences of closely related species were retrieved from GenBank/EMBL/DDBJ. The neighbour-joining, maximum-likelihood and maximum-parsimony methods were used to construct phylogenetic trees as described previously (Endo & Okada, 2006). Approximately 1400 bp of the 16S rRNA gene sequence of each *L. kunkeei* isolate and those of related species were used to construct the phylogenetic trees. The 16S rRNA gene sequence similarity between the eight new isolates was over 99.9% and they shared more than 99.8% gene sequence similarity with *L. kunkeei* DSM 12361^T. None of the other related species shared 16S rRNA gene sequence similarities higher than 95% with the eight new isolates. The nine isolates, including *L. kunkeei* DSM 12361^T, formed an independent subcluster in the *Lactobacillus* phylogenetic group when analysed using the

neighbour-joining method (Fig. 1). Identical tree topologies were obtained by using the maximum-likelihood and maximum-parsimony analyses (data not shown).

The levels of DNA–DNA relatedness between the eight isolates and *L. kunkeei* DSM 12361^T, and the DNA G+C contents of the nine isolates were determined according to the methods described by Endo & Okada (2006). Extraction and purification of bacterial DNA was performed according to the method of Marmur (1961), as modified by Ezaki *et al.* (1983). The eight isolates showed high levels of DNA–DNA relatedness to *L. kunkeei* DSM 12361^T (ranging from 77 to 99%). Based on this result, it was concluded that the eight new isolates were additional strains of *L. kunkeei*. The DNA G+C contents of the nine isolates ranged from 36 to 37%.

Morphological, physiological and biochemical characteristics of the nine isolates were determined using previously described methods (Endo *et al.*, 2009). Detailed characteristics of the isolates are given in the species description. The eight isolates produced acids from only four or five of the carbohydrates tested (Table 2). The isolates fermented fructose faster than glucose and produced acids from fructose within 1 to 2 days. The production of acids from glucose took 3 to 4 days. This is a typical characteristic of ‘obligately’ fructophilic lactic acid bacteria. Strain DSM 12361^T fermented eight carbohydrates. Glucose fermentation took 5 days and fructose fermentation took 7 days. All nine isolates produced acids from sucrose and most isolates fermented mannitol and trehalose.

The growth characteristics of the *L. kunkeei* isolates were determined as described previously (Endo *et al.*, 2009). All of the isolates grew well on fructose but poorly on glucose (see Supplementary Fig. S1a and b in IJSEM Online). However, the isolates grew very well on glucose in the presence of 1% pyruvate or under aerobic conditions with shaking (Supplementary Fig. S1b), suggesting that the isolates needed external electron acceptor(s) for the metabolism of glucose. This correlated well with the definition of ‘obligately’ fructophilic lactic acid bacteria (Endo *et al.*, 2009, 2011). In contrast, strain DSM 12361^T grew very slowly on glucose and on fructose (Supplementary Fig. S1a). The strain took 5–7 days to grow on both substrates. Growth of the type strain DSM 12361^T on

Table 1. List of *L. kunkeei* strains used in this study

Strain	Origin (country)	Reference	16S rRNA gene sequence GenBank accession no.
DSM 12361 ^T	Red wine (USA)	Edwards <i>et al.</i> (1998)	Y11374
IWB T B226	Wine (South Africa)	Bayly (1999)	AB559818
IWB T B227	Wine (South Africa)	Bayly (1999)	AB559819
F7-2	Azalea (South Africa)	Endo <i>et al.</i> (2009)	AB495039
F10-3	Narcissus (South Africa)	Endo <i>et al.</i> (2009)	AB498040
F20-1	Cosmos (South Africa)	Endo <i>et al.</i> (2009)	AB498042
NRIC 0776	Honey (Japan)	This study	AB559820
NRIC 0777	Morning glory (Japan)	This study	AB559821
NRIC 0778	Crape myrtle (Japan)	This study	AB559822

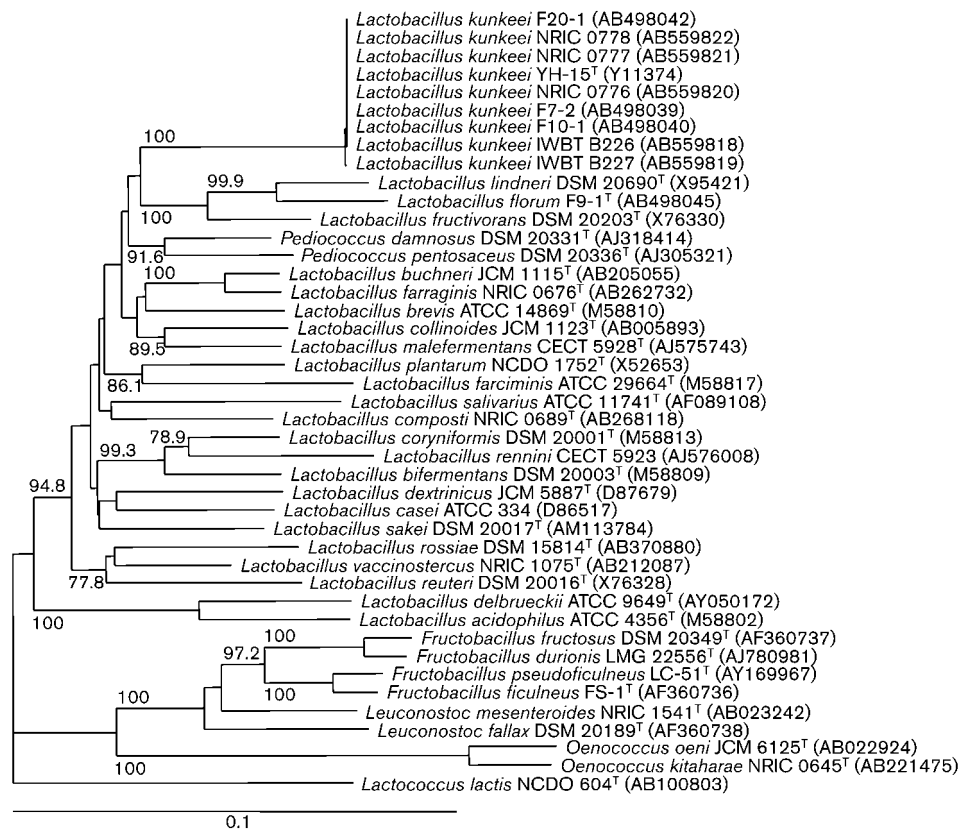


Fig. 1. Phylogenetic relationship of *L. kunkeei* isolates to related species based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method. *Lactococcus lactis* NCDO 604^T was used as an outgroup. Bootstrap percentages above 70% are given at branching points. Bar, 0.1 substitutions per nucleotide position.

glucose was enhanced in the presence of pyruvate or under aerobic conditions (Supplementary Fig. S1a). Interestingly, although the type strain grew very slowly on glucose or on fructose, the strain grew well in co-fermentation of glucose

Table 2. Acid production from carbohydrates for strains of *L. kunkeei*

Strains: 1, DSM 12361^T; 2, IWB T B226; 3, IWB T B227; 4, F7-2; 5, F10-3; 6, F20-1; 7, NRIC 0776; 8, NRIC 0777; 9, NRIC 0778. Values are the number of days needed for fermentation. –, Negative result; w, weak reaction.

Characteristic	1	2	3	4	5	6	7	8	9
Fructose	7	2	1	1	1	1	1	2	1
Galactose	7	–	–	–	–	–	–	–	–
Glucose	5	4	3	3	3	3	3	3	3
Mannitol	8	–	w	6	w	5	–	w	w
Melibiose	8	–	–	–	–	–	–	–	–
Potassium gluconate	w	w	w	–	w	w	w	w	w
Sucrose	6	3	3	2	2	2	3	3	3
Trehalose	8	–	3	–	3	3	–	3	3

and fructose. Mannitol was produced from fructose. This means fructose was used as an electron acceptor in the co-fermentation. Moreover, whereas strain DSM 12361^T grew well on glucose under aerobic conditions, it grew weakly on fructose under aerobic conditions (Supplementary Fig. S1a), suggesting that fructose is very weakly fermented. The possible reason for these interesting results may be attributed to the phosphoenolpyruvate-sugar phosphotransferase (sugar PTS) of fructose. Lactobacilli usually have two systems for the uptake of fructose, which are fructose-PTS for utilization of fructose as a substrate and fructose permease for utilization of fructose as an electron acceptor (Akinterinwa *et al.*, 2008). A fructose/mannitol-specific transporter is usually found in several lactobacilli (Taranto *et al.*, 1999). As strain DSM 12361^T used fructose as an electron acceptor but weakly as the substrate, the strain may have a very weak fructose/mannitol-specific transporter activity for PTS. Mannitol dehydrogenase, which acts as an electron acceptor for fructose (Liu *et al.*, 2005; Sasaki *et al.*, 2005), is not related to PTS (Akinterinwa *et al.*, 2008). All *L. kunkeei* isolates produced gas from D-glucose, but produced almost equimolar amounts of lactic acid and acetic acid, and very little ethanol (at a molar ratio of 1:0.9–1.2:0.005–0.01, respectively). These characteristics are unique in the group of obligately heterofermentative lactic acid

bacteria, but correlated well with the characteristics of 'obligately' fructophilic lactic acid bacteria, including *Fructobacillus* species (Endo & Okada, 2008; Endo *et al.*, 2009).

All nine isolates, including the type strain, grew well on MRS agar under aerobic conditions, and colonies on MRS agar were approximately 1–2 mm in diameter after 2 days of incubation (Supplementary Fig. S2a). However, the isolates grew weakly on MRS agar under anaerobic conditions provided by means of a gas generating kit (Anaerobic System BR0038B; Oxoid Ltd), and colony sizes were approximately 0.1–0.2 mm in diameter after incubation for 2 days (Supplementary Fig. S2b). These characteristics were not in accordance with the usual characteristics of the genus *Lactobacillus*, but were consistent with those of fructophilic lactic acid bacteria (Endo *et al.*, 2009, 2011).

In conclusion, the eight new isolates of *L. kunkeei* were found to be typical 'obligately' fructophilic lactic acid bacteria. As far as the authors are aware, this is the first study to demonstrate the presence of fructophilic lactic acid bacteria in wine. Strain DSM 12361^T appeared to be an atypical strain of the species and revealed slightly different characteristics when compared with the eight other isolates. However, the type strain showed several typical fructophilic characteristics, such as enhanced growth on glucose in the presence of electron acceptors, including fructose, and little ethanol production from glucose. These are important characteristics of fructophilic lactic acid bacteria. It is thus safe to conclude that *L. kunkeei* belongs to the group of 'obligately' fructophilic lactic acid bacteria. Several characteristics not presented in the original description of *L. kunkeei* were investigated in the present study and an emended description of the species is proposed.

Emended description of *Lactobacillus kunkeei* Edwards *et al.* 1998

Lactobacillus kunkeei (N.L. gen. n. *kunkeei* of Kunkee, named after Ralph E. Kunkee, University of California, Davis, CA, USA, for his contributions to the microbiology of wines).

Cells are Gram-positive, non-motile rods approximately $0.5 \times 1\text{--}1.5$ μm . Cells occur singly or in pairs and in chains. Facultatively anaerobic and catalase-negative, but catalase activity is found in cells grown in the presence of haem. Obligately fructophilic lactic acid bacteria and external electron acceptors, such as pyruvate, oxygen and fructose, enhance growth on glucose. Colonies on MRS agar are white, smooth and approximately 1–2 mm in diameter under aerobic conditions, but approximately 0.1–0.2 mm in diameter under anaerobic conditions after incubation for 2 days at 30 °C. In liquid broth, growth on glucose is very weak, but, usually (except for the type strain), growth on fructose is rapid. External electron acceptors such as pyruvate and oxygen enhance the growth of the strains. The strains produce almost equimolar amounts of lactic acid and acetic acid and trace amounts of ethanol. D- and L-lactic acid are produced at a ratio of 2 : 8. Gas is produced

from glucose. Mannitol is produced from fructose. Nitrate is not reduced. Ammonia is not formed from arginine. Acid is produced from D-fructose, D-glucose and sucrose. Acid production from D-galactose, mannitol, melibiose, trehalose and potassium gluconate (weakly) are strain dependent. 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 -gentiobiose, 2- and 5-ketogluconate, methyl α -D-glucoside, glycerol, glycogen, inositol, inulin, D-lyxose, D-mannose, methyl α -D-mannoside, melezitose, raffinose, rhamnose, salicin, starch, sorbitol, L-sorbose, D-tagatose, turanose, xylitol, L-xylose, methyl β -xyloside, lactose or D-xylose. Dextran is not produced from sucrose. Strains grow at 15 °C, but not at 45 °C. Cells usually grow at pH 4.5–6.8 (5.5–6.8 for the type strain). The cell-wall peptidoglycan is A4 α type (L-Lys–D-Asp). The DNA G + C content ranges from 36 to 37 mol%.

The type strain is YH-15^T (=ATCC 700308^T=DSM 12361^T). The species inhabits fructose-rich niches, e.g. wine, flowers, honey and fruits.

References

- Akinterinwa, O., Khankal, R. & Cirino, P. C. (2008). Metabolic engineering for bioproduction of sugar alcohols. *Curr Opin Biotechnol* **19**, 461–467.
- Bayly, J. C. (1999). The isolation, identification and characterization of lactic acid bacteria occurring in South African red wine fermentations. BSc Hons thesis, Stellenbosch University, Stellenbosch.
- Edwards, C. G., Haag, K. M., Collins, M. D., Hutson, R. A. & Huang, Y. C. (1998). *Lactobacillus kunkeei* sp. nov.: a spoilage organism associated with grape juice fermentations. *J Appl Microbiol* **84**, 698–702.
- Endo, A. & Okada, S. (2005). *Lactobacillus satsumensis* sp. nov., isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials. *Int J Syst Evol Microbiol* **55**, 83–85.
- Endo, A. & Okada, S. (2006). *Oenococcus kitaharae* sp. nov., a non-acidophilic and non-malolactic-fermenting oenococcus isolated from a composting distilled shochu residue. *Int J Syst Evol Microbiol* **56**, 2345–2348.
- Endo, A. & Okada, S. (2008). Reclassification of the genus *Leuconostoc* and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. *Int J Syst Evol Microbiol* **58**, 2195–2205.
- Endo, A., Futagawa-Endo, Y. & Dicks, L. M. T. (2009). Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. *Syst Appl Microbiol* **32**, 593–600.
- Endo, A., Futagawa-Endo, Y., Sakamoto, M., Kitahara, M. & Dicks, L. M. T. (2010). *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. *Int J Syst Evol Microbiol* **60**, 2478–2482.
- Endo, A., Irisawa, T., Futagawa-Endo, Y., Sonomoto, K., Itoh, K., Takano, K., Okada, S. & Dicks, L. M. T. (2011). *Fructobacillus tropaeoli* sp. nov., a novel fructophilic lactic acid bacterium isolated from a flower. *Int J Syst Evol Microbiol* **61**, 898–902.
- Ezaki, T., Yamamoto, N., Ninomiya, K., Suzuki, S. & Yabuuchi, E. (1983). Transfer of *Peptococcus indolicus*, *Peptococcus asaccharolyticus*,

and *Peptococcus magnus* to the genus *Peptostreptococcus* and proposal of *Peptostreptococcus tetradius* sp. nov. *Int J Syst Bacteriol* **33**, 683–698.

Liu, S., Saha, B. & Cotta, M. (2005). Cloning, expression, purification, and analysis of mannitol dehydrogenase gene *mtlK* from *Lactobacillus brevis*. *Appl Biochem Biotechnol* **121**, 391–401.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Sasaki, Y., Laivenieks, M. & Zeikus, J. G. (2005). *Lactobacillus reuteri* ATCC 53608 *mdh* gene cloning and recombinant mannitol dehydrogenase characterization. *Appl Microbiol Biotechnol* **68**, 36–41.

Taranto, M. P., Font de Valdez, G. & Perez-Martinez, G. (1999). Evidence of a glucose proton motive force-dependent permease and a fructose phosphoenolpyruvate:phosphotransferase transport system in *Lactobacillus reuteri* CRL 1098. *FEMS Microbiol Lett* **181**, 109–112.