Lactobacillus taiwanensis sp. nov., isolated from silage

Li-Ting Wang, Hsiao-Ping Kuo, Yen-Chi Wu, Chun-Ju Tai and Fwu-Ling Lee

Correspondence
Fwu-Ling Lee
fll@firdi.org.tw

Bioresource Collection and Research Center, Food Industry Research and Development Institute, PO Box 246, Hsinchu 30099, Taiwan, ROC

Strain FIRDI 006^T, isolated from silage cattle feed, was characterized by using phenotypic and molecular taxonomic methods. The strain was a homofermentative lactic acid bacterium; the cells stained Gram-positive and were catalase-negative, non-motile, facultatively anaerobic rods. 16S rRNA gene sequence analysis revealed that the strain belongs phylogenetically to the genus *Lactobacillus* and can be placed within the *Lactobacillus acidophilus*–*delbrueckii* group. *Lactobacillus gasseri* and *Lactobacillus johnsonii* were the most closely related species, with 16S rRNA gene sequence similarities of 99.53 and 99.46 % to the respective type strains. Low *gyrB* gene sequence similarities (<90 %) and low DNA–DNA reassociation values (<45 %) were obtained between the strain and the phylogenetically closest neighbours. Based on phenotypic and genetic evidence, the strain is considered to represent a novel species, for which the name *Lactobacillus taiwanensis* sp. nov. is proposed. The type strain is FIRDI 006^T (=BCRC 17755^T =DSM 21401^T).

Lactobacillus acidophilus is a Gram-positive, non-sporulating, rod-shaped, catalase-negative, non-motile, homofermentative and facultatively anaerobic bacterium (Hansen & Møcquot, 1970). Its health benefits make it one of the most important probiotic species (Metchnikoff, 1908; Huis in't Veld, 1992; Ferencik *et al.*, 2000; Taranto *et al.*, 2004). The *Lactobacillus acidophilus* species group is composed of *L. acidophilus* and its phylogenetically closest relatives (Felix & Dellaglio, 2007; Naser *et al.*, 2007). Some of these species are quite difficult to distinguish by phenotypic characteristics, but can be differentiated by DNA–DNA hybridization, gene sequence analysis and DNA typing (Johnson *et al.*, 1980; Lauer *et al.*, 1980; Berger *et al.*, 2007; Naser *et al.*, 2007). A polyphasic taxonomic study of the members of the *L. acidophilus* species group and a novel strain, FIRDI 006^T, was performed using phenotypic characterization and phylogenetic and genetic methods. The results of this study consistently indicate that strain FIRDI 006^T represents a novel *Lactobacillus* species.

Strain FIRDI 006^T was isolated from silage cattle feed taken from Jhunan, Miaoli, Taiwan in 2006. For isolation of strain FIRDI 006^T, a 10 g forage sample was diluted in

MRS broth, plated onto MRS agar (Difco) and incubated at 37 °C for 2–3 days under anaerobic conditions (Genbox anaer; bioMérieux). Morphologically distinct single colonies that developed were picked up and restreaked onto the same medium at least twice before being considered pure. The pure strain was stored long-term at –80 °C in MRS broth containing 20 % (v/v) glycerol or preserved by lyophilization. In addition to strain FIRDI 006^T, the reference strains *L. acidophilus* BCRC 10695^T, *Lactobacillus helveticus* BCRC 12936^T, *Lactobacillus crispatus* BCRC 14618^T, *Lactobacillus gasseri* BCRC 14619^T, *Lactobacillus amylovorus* BCRC 11648^T, *Lactobacillus johnsonii* BCRC 17474^T, *Lactobacillus gallinarum* BCRC 17266^T, *Lactobacillus amyolyticus* BCRC 17497^T and *Lactobacillus kitasatonis* BCRC 17512^T were selected for further comparative study.

Chromosomal DNA was isolated using the Qiagen Blood & Cell Culture DNA kit. The 16S rRNA gene sequence was amplified by PCR and sequenced using the MicroSeq Full Gene 16S rDNA Bacterial Identification kit (Applied Biosystems). Sequencing was performed with a 3730 DNA sequencer (Applied Biosystems and Hitachi). Sequence assembly was performed using the ABI PRISM DNA Sequencing Analysis software (PE Applied Biosystems). Sequence similarity was calculated using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The sequences obtained in this study and from GenBank were aligned using the CLUSTAL_X program, version 1.8 (Thompson *et al.*, 1997), and phylogenetic trees were constructed with the neighbour-joining, maxi-

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain BCRC 17755^T (=FIRDI 006^T) are respectively EU487512 and EU647675.

Neighbour-joining and maximum-parsimony phylogenetic trees based on 16S rRNA and *gyrB* gene sequences of the novel strain and related species and detailed results of DNA–DNA hybridization studies are available as supplementary material with the online version of this paper.

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences of *Lactobacillus* strains. Bar, 10% nucleotide substitutions.

mum-parsimony and maximum-likelihood methods using the PHYLIP computer program package (Felsenstein, 2005). Sequence distances were calculated by the F84 model (Felsenstein, 1984). Bootstrap analysis was made with 1000 replicates (Fig. 1 and Supplementary Figs S1 and S2, available in IJSEM Online). Tree figures were drawn by TreeView. The results of the phylogenetic analysis indicated that strain FIRDI 006^T belongs to the genus *Lactobacillus* and is closely related to the *Lactobacillus acidophilus*–*delbrueckii* group (Fig. 1). It has been reported

that six genome clusters (A1–A4, B1 and B2) exist within the *L. acidophilus* species group (Johnson *et al.*, 1980; Lauer *et al.*, 1980). Homology groups B1 and B2 were designated *L. gasseri* and *L. johnsonii* by Lauer & Kandler (1980) and Fujisawa *et al.* (1992). Strain FIRDI 006^T was included in a monophyletic cluster that contained *L. gasseri* and *L. johnsonii*. The highest levels of sequence similarity were with the type strains of *L. gasseri* (99.53%) and *L. johnsonii* (99.46%). The lowest level of sequence similarity was with the type strain of *Lactobacillus iners* (95.14%).

Fig. 2. Maximum-likelihood tree based on *gyrB* gene sequences of *Lactobacillus* strains. Bar, 10% nucleotide substitutions.

To differentiate further between strain FIRDI 006^T and closely related species, the partial sequence for the gene encoding the subunit B protein of DNA gyrase (*gyrB*) was included in the phylogenetic analysis for comparative purposes. The *gyrB* gene was amplified by PCR as described previously (Yamamoto & Harayama, 1995). The PCR was performed using a *Taq* DNA polymerase kit (Invitrogen). PCR products were purified with the PCR-M clean up system (Viogene) and sequenced with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). DNA sequencing was determined using degenerate *gyrB* primers for strain FIRDI 006^T, UP-1S and LA900R (5'-CCTGGCAARTTDGCAATTTTC-3') and, for other related *Lactobacillus* species, primers UP-1S, LA900R and UP-2Sr (Yamamoto & Harayama, 1995). Sequence similarity was calculated using the program EMBOSS-Align (Needleman–

Wunsch global alignment) (<http://www.ebi.ac.uk/>). Using the method described above, the sequences obtained in this study were also aligned and phylogenetic trees were constructed (Fig. 2 and Supplementary Figs S3 and S4). The tree based on 777 bp *gyrB* gene sequences showed clearly that strain FIRDI 006^T formed a branch. Strain FIRDI 006^T shared 87.15 and 90.09 % similarity with *L. johnsonii* BCRC 17474^T and *L. gasseri* BCRC 14619^T, whereas the similarities between strain FIRDI 006^T and other members of the related cluster were less than 83 %. The *gyrB* gene sequence was a reliable indicator that the isolate was significantly differentiated from *L. johnsonii* and *L. gasseri*.

DNA G+C contents and DNA–DNA relatedness were determined as described previously (Tamaoka &

Table 1. Differentiating characteristics for strain FIRDI 006^T (*Lactobacillus taiwanensis* sp. nov.) and its phylogenetically closest relatives

Strains: 1, FIRDI 006^T (*L. taiwanensis* sp. nov.); 2, *L. johnsonii* BCRC 17474^T; 3, *L. gasseri* BCRC 14619^T; 4, *L. acidophilus* BCRC 10695^T; 5, *L. crispatus* BCRC 14618^T; 6, *L. helveticus* BCRC 12936^T; 7, *L. gallinarum* BCRC 17266^T; 8, *L. amylovorus* BCRC 11648^T; 9, *L. kitasatonis* BCRC 17512^T; 10, *L. amylolyticus* BCRC 17497^T. DNA G+C contents for *L. gallinarum*, *L. amylovorus* and *L. kitasatonis* are ranges for several strains taken from Mukai *et al.* (2003); other data were obtained in this study. All strains grew at 45 °C and at pH 6.5 and 8.0. All strains grew in medium with 2 % NaCl, but not with 7 % NaCl. None of the strains produced gas from glucose. All strains produced acid from D-glucose, D-fructose and maltose. None of the strains produced acid from D- or L-arabinose, D- or L-fucose, D-lyxose, melezitose, L-rhamnose, D-ribose, L-sorbose, turanose, D- or L-xylose, inulin, D-adonitol, D- or L-arabitol, dulcitol, erythritol, glycerol, inositol, D-mannitol, D-sorbitol, xylitol, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate.

Characteristic	1	2	3	4	5	6	7	8	9	10
Growth in MRS broth at/in:										
15 °C	–	+	–	–	–	–	–	–	–	–
pH 4.0	+	+	+	–	+	–	–	+	+	+
5 % NaCl	+	+	+	–	–	–	–	–	–	–
Gas production from gluconate	–	–	+	+	–	–	–	–	–	–
Acid production from:										
D-Galactose	+	+	+	–	–	+	+	+	+	–
D-Mannose	+	+	+	+	–	+	+	+	–	+
N-Acetylglucosamine	–	+	+	+	+	+	+	+	+	+
Amygdalin	–	–	+	+	+	–	+	–	–	–
Arbutin	–	–	+	–	–	–	–	+	–	–
Aesculin	+	+	+	+	+	–	+	+	+	–
Salicin	–	–	+	+	+	–	+	+	–	–
Cellobiose	–	–	+	+	+	–	+	+	+	–
Lactose	+	+	+	+	+	+	+	–	–	–
Melibiose	–	–	–	–	–	–	+	–	–	–
Sucrose	+	+	+	+	+	–	+	+	+	+
Trehalose	+	–	+	+	–	+	–	+	–	–
Raffinose	–	+	–	–	–	–	+	–	–	–
Starch	+	+	+	–	+	–	+	+	+	–
Glycogen	–	–	–	–	+	–	+	+	–	–
Gentiobiose	+	+	+	+	–	–	+	+	+	–
D-Tagatose	–	+	+	–	–	–	–	–	–	–
DNA G+C content (mol%)	35.8	35.9	35.8	35.7	39.1	39.2	36–37	36–38	37–40	40.1
Sequence similarity (%) with respect to strain FIRDI 006 ^T										
16S rRNA gene	(100)	99.46	99.53	92.54	92.67	93.00	93.14	92.34	92.56	92.34
<i>gyrB</i>	(100)	87.15	90.09	82.88	81.21	81.60	83.91	82.65	83.27	82.24

Komagata, 1984; Ezaki *et al.*, 1989; Goris *et al.*, 1998; Wang *et al.*, 2007). The DNA G + C content of strain FIRDI 006^T was 35.8 mol%. This value is close to the values of 35.9 and 35.8 mol% for *L. johnsonii* BCRC 17474^T and *L. gasseri* BCRC 14619^T (Table 1). Using labelled DNA of strain FIRDI 006^T and *L. johnsonii* BCRC 17474^T, low levels of reassociation (<45 %) were observed with the type strains of closely related *Lactobacillus* species and subspecies (Supplementary Table S1). These values are well below the threshold of 70 % that has been suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that strain FIRDI 006^T represents a separate genomic species.

Morphological, physiological and biochemical characteristics of all strains were determined by using cells grown on MRS agar plates or in MRS broth for 2 days at 37 °C under anaerobic conditions. The characteristics of strain FIRDI 006^T (described in detail in the species description) were compared with those of type strains of closely related species (Table 1). All of the strains grew at 45 °C but not at 15 °C except *L. johnsonii* BCRC 17474^T. Strain FIRDI 006^T, *L. johnsonii* BCRC 17474^T and *L. gasseri* BCRC 14619^T grew at pH 4.0, 6.5 and 8.0 and in medium containing 2 and 5 % (w/v) NaCl. Strain FIRDI 006^T did not produce gas from glucose or gluconate, as examined with Durham tubes; the absence of CO₂ production could indicate either an obligately homofermentative or a facultatively heterofermentative metabolism (Hammes & Vogel, 1995). Carbohydrate fermentation patterns were evaluated using the API 50 CHL system (bioMérieux) according to the manufacturer's instructions. The results were recorded after 48 h at 37 °C. All strains were found to display similar fermentation characteristics.

Based on the reported data, the phenotypic characteristics of the isolate were similar to those of its phylogenetically closest relatives, *L. gasseri* and *L. johnsonii*, and other homofermentative *Lactobacillus* species (Table 1). However, *gyrB* gene sequence similarity and DNA–DNA relatedness showed a clear separation of strain FIRDI 006^T from its closest phylogenetic relatives. Thus, we propose the novel species *Lactobacillus taiwanensis* sp. nov. to accommodate strain FIRDI 006^T.

Description of *Lactobacillus taiwanensis* sp. nov.

Lactobacillus taiwanensis (tai.wa.nen'sis. N.L. masc. adj. *taiwanensis* of Taiwan, referring to the geographical origin of the type strain).

Cells are Gram-positive-staining, non-motile, non-spore-forming, catalase-negative rods, 0.5–1.0 × 2.0–20.0 µm, occurring as single cells or in pairs or chains. Grows better at 37 °C than at 30 °C. Colonies are approximately 2 mm in diameter, circular to slightly irregular, convex, opaque, rough and white after 2 days at 37 °C on MRS agar plates. Facultatively anaerobic and produces DL-lactic acid homofermentatively. In MRS broth, growth occurs at 45 °C but not at 15 or 50 °C. Growth is observed in MRS broth containing 2 or 5 % (w/v) NaCl and at pH 4–8. Acid is

produced without gas formation from D-glucose, D-galactose, D-fructose, D-mannose, maltose, lactose, sucrose, trehalose, gentiobiose, starch and aesculin. No acid formation from D- or L-arabinose, cellobiose, D- or L-fucose, D-lyxose, melibiose, melezitose, raffinose, D-ribose, L-rhamnose, L-sorbose, D-tagatose, turanose, D- or L-xylose, N-acetylglucosamine, inulin, D-adonitol, D- or L-arabitol, dulcitol, erythritol, glycerol, inositol, D-mannitol, D-sorbitol, xylitol, glycogen, amygdalin, arbutin, salicin, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The DNA G + C content of the type strain is 35.8 mol%.

The type strain, FIRDI 006^T (=BCRC 17755^T =DSM 21401^T), was isolated from silage cattle feed in Taiwan in 2006.

Acknowledgements

We thank T. Y. Liu, C. C. Liao and G. F. Yuan (Food Industry Research and Development Institute, Taiwan) for their encouragement. This research was supported by the Council of Agriculture, Executive Yuan, ROC [project no. 96AS-3.1.1-AD-U1(3)].

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