**Lactococcus formosensis** sp. nov., a lactic acid bacterium isolated from *yan-tsai-shin* (fermented broccoli stems)

Yi-sheng Chen,1 Misa Otoguro,2 Yu-hsuan Lin,1 Shwu-fen Pan,1 Si-hua Ji,1 Chi-rong Yu,1 Min-shiuan Liou,1 Yu-chung Chang,1 Hui-chung Wu1 and Fujitoshi Yanagida2

1Department of Biotechnology, Ming Chuan University, No. 5, De-Ming Road, Gui-Shan Township, Taoyuan County 333, Taiwan, ROC
2The Institute of Enology and Viticulture, University of Yamanashi, 1-13-1, Kitashin, Kofu, Yamanashi 400-0005, Japan

A coccal-shaped organism, designated 516T, was isolated from *yan-tsai-shin* (fermented broccoli stems), a traditional fermented food in Taiwan. 16S rRNA gene sequencing results showed that strain 516T had 98.9% sequence similarity to that of the type strain *Lactococcus garvieae* NBRC 100934T. Comparison of three housekeeping genes, *rpoA*, *rpoB* and *pheS*, revealed that strain 516T was well separated from *Lactococcus garvieae* NBRC 100934T. DNA–DNA hybridization studies indicated that strain 516T had low DNA relatedness with *Lactococcus garvieae* NBRC 100934T (46.1%). The DNA G+C content of strain 516T was 38.1 mol% and the major fatty acids were C16 : 0 (22.7%), C19 : 0 cyclo (17.9%) and summed feature 7 (29.0%). Based on the evidence, strain 516T represents a novel species of the genus *Lactococcus*, for which the name *Lactococcus formosensis* sp. nov. is proposed. The type strain is 516T (=NBRC 109475T) = BCRC 80576T.

The genus *Lactococcus* currently contains eight species, and four subspecies have been further established within the species *Lactococcus lactis* (Cai et al., 2011; Pérez et al., 2011; Chen et al., 2013). Species of the genus *Lactococcus* have been previously isolated from various food, plant and animal sources (Williams et al., 1990; Cai et al., 2011; Pérez et al., 2011; Chen et al., 2013). Lactic acid bacteria including the species of the genus *Lactococcus*, are widely distributed in traditional fermented foods in Taiwan. In our previous studies, we identified a novel species of the genus *Lactobacillus* named *Lactobacillus pobuzii* in pobuzihi (fermented cumming cordia) and a novel species of the genus *Lactococcus* named *Lactococcus taiwanensis* in its raw material, fresh cumming cordia (Chen et al., 2010, 2013). Besides pobuzihi, a survey on the distribution of lactic acid bacteria in *yan-tsai-shin* (fermented broccoli stems) was performed in our recent study. All isolates obtained from *yan-tsai-shin* were identified based on their phenotypic and phylogenetic characteristics. The 16S rRNA gene sequence of strain 516T showed 98.9% sequence similarity to that of the type strain of *Lactococcus garvieae*, NBRC 100934T. However, differences in nucleotide sequences at several specific positions were observed between strain 516T and *Lactococcus garvieae* NBRC 100934T. In order to exactly identify strain 516T, analysis of three housekeeping genes, *rpoA*, *rpoB* and *pheS* (Pérez et al., 2011; Rahkila et al., 2012) and phenotypic characteristics was performed. The purpose of the present study was to establish the taxonomic position of this bacterial strain.

Strain 516T is a Gram-stain-positive and catalase-negative coccus. Tests of phenotypic characteristics, such as isomers of lactic acid produced, lactic acid fermentation type, salt tolerance and growth temperature range, were carried out based on previously described procedures (Chen et al., 2006, 2010). Tests of acid production from carbohydrates were performed using the API 50 CHL fermentation kit (bioMérieux) according to the manufacturer’s instructions. In addition, the enzymic activities of strain 516T and *Lactococcus garvieae* NBRC 100934T were assayed using the API ZYM system (bioMérieux). The tests were performed according to the manufacturer’s instructions. After incubation at 37 °C for 4 h, the reaction was terminated by the addition of one drop each of A and B API ZYM reagents and the results were examined.

**Abbreviation:** NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoA*, *rpoB* and *pheS* gene sequences of strain 516T are AB775178, AB780470, AB775180 and AB775179, respectively.

Eight supplementary figures and two supplementary tables are available with the online version of this paper.
Amplification and sequencing of the 16S rRNA gene was carried out as described by Chen et al. (2006). Amplification and sequencing of the housekeeping genes rpoA, rpoB and pheS were performed using primers rpoA-21-F (5′-AT-GATYGARTTGGAAAACC-3′), rpoA-23-R (5′-ACGTRTTRATDCCDGCRG-3′), rpoBLac1F (5′-TAGGGKAACACCGTA-3′), rpoBLac1R (5′-TCAARCCAWGCTCCACGG-3′), pheS-21-F (5′-CAYCCNGCHCGYGAYATGC-3′) and pheS-22-R (5′-CCWARVCCRAARGCAAARC-3′), which were previously designed and reported by Pérez et al. (2011) and Naser et al. (2005). PCRs were carried out using a Genomics Taq gene amplification PCR kit (Genomics) and performed on a Gene Amp PCR System 9700 (PerkinElmer) following the methods described by Chen et al. (2006), Pérez et al. (2011) and Naser et al. (2005). DNA sequencing was performed using an ABI 3730 DNA Analyser (Applied Biosystems). Sequence homologies were determined using BLAST (Altschul et al. 1997) against the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using BLAST (Altschul et al. 1997).

All sequences were aligned using CLUSTAL W software (Thompson et al., 1997). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were then reconstructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987), with bootstrap analysis based on 1000 iterations. The MEGA 5.05 package (Tamura et al., 2007) was used for all analyses.

Genomic DNA was extracted from cells grown in MRS broth for 24 h at 30 °C and purified using the Qiagen Blood & Cell Culture DNA kit (Qiagen) (Aguado-Urda et al., 2011). The DNA G+C content was determined using reversed-phase HPLC as described previously (Tamaoka & Komagata, 1984; Wang et al., 2007). DNA–DNA relatedness values were determined using the fluorometric hybridization method in microdilution wells as described previously (Ezaki et al., 1989; Goris et al., 1998; Wang et al., 2007).

Nucleotide sequences of 16S rRNA (approximately 1515 nt), rpoA (approximately 817 nt), rpoB (approximately 494 nt) and pheS (approximately 510 nt) genes were determined. Phylogenetic analysis of the 16S rRNA gene sequences obtained in this study and from GenBank indicated that strain 516T belonged to the genus Lactococcus. Strain 516T formed a monophyletic cluster with the type strains of species of the genus Lactococcus, supported by a bootstrap value of 100% (Fig. 1). This novel strain showed the highest sequence similarity (98.9%) to Lactococcus garvieae NBRC 100934T and the second highest similarity of 92.4% to Lactococcus fujisensis NJ 317T.

When comparing the rpoA gene sequences with those held in the GenBank database, strain 516T showed the highest similarity (97.7%) to Lactococcus garvieae NBRC 100934T and the second highest similarity (84.5%) to Lactococcus lactis subsp. cremoris LMG 6897T. The phylogenetic analysis of the rpoA gene is shown in Fig. 2.

Comparison of the rpoB gene with that of other type strains of species of the genus Lactococcus was also performed. The results indicated that strain 516T has 96.9% sequence similarity to Lactococcus garvieae NBRC 100934T. The phylogenetic analysis of the rpoB gene is shown in Fig. 3.

The pheS gene of strain 516T was also compared with those of type strains of other species of the genus Lactococcus. The results showed that strain 516T has 91.5% sequence similarity to Lactococcus garvieae NBRC 100934T. The phylogenetic analysis of the pheS gene is shown in Fig. 4.

The topology was also analysed using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971, 1977) methods. Bootstrap analyses were made

![Fig. 1. Neighbour-joining tree of Lactococcus formosensis sp. nov. 516T and other related lactococci based on 16S rRNA sequences. Bootstrap values are indicated at branch points based on 1000 iterations. GenBank accession numbers are given in parentheses. The 16S rRNA sequence of Lactococcus garvieae NBRC 100934T were obtained from the NBRC (NITE Biological Resource Center) database. Bacillus subtilis NCDO 1769T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
with 1000 iterations. Similar results were obtained to those for the NJ method (Figs 1–4 and Figs. S1–S8, available in IJSEM Online).

Besides phylogenetic analyses, a number of phenotypic tests were performed. Strain 516T showed homolactic acid fermentation, production of L-lactic acid and growth in a medium with 6 % (w/v) NaCl. The tolerance to 6 % NaCl clearly differentiated strain 516T from \textit{Lactococcus garvieae} NBRC 100934T (Table 1). Besides, \textit{Lactococcus garvieae} NBRC 100934T grew well at pH 5.0 but strain 516T did not (Table 1). Acid production from carbohydrates was assessed using the API 50 CHL fermentation kit after 48 h of incubation; unexpectedly, identical results to those for \textit{Lactococcus garvieae} NBRC 100934T were observed (Table 1). In addition, the enzymic activities evaluated by API ZYM system are shown in Table S1. Strain 516T showed similar enzyme profiles to \textit{Lactococcus garvieae} NBRC 100934T and only a small difference was observed, regarding the enzymic activity of \(\alpha\)-fucosidase (Table S1).

The DNA G+C content of strain 516T was 38.1 mol\%, which was similar to that of \textit{Lactococcus garvieae} NBRC 100934T (38.3–38.7 mol\%) (Table 1). DNA–DNA hybridization experiments were performed using DNA derived from strain 516T (probe) and \textit{L. garvieae} NBRC 100934T (target). DNA–DNA relatedness values of strain 516T with \textit{Lactococcus garvieae} NBRC 100934T was 46.1 %, indicating that strain 516T does not meet the threshold for inclusion as a representative of \textit{Lactococcus garvieae}.

Biomass for chemotaxonomic studies was obtained by growing the isolate under aerobic conditions in MRS broth for 2 days at 37 °C and harvesting by centrifugation. Cell-wall samples were prepared from approximately 1 g wet cell weight by mechanical disruption with an ultrasonic oscillator and were purified as described by Schleifer & Kandler (1972). The amino acids in cell-wall hydrolysates were analysed using the methods described by Hamada et al. (2010). Extraction and determination of cellular fatty acid profiles were performed by using the Sherlock

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**Fig. 2.** Neighbour-joining tree of \textit{Lactococcus formosensis} sp. nov. 516\textsuperscript{T} and other related lactococci based on \textit{rpoA} sequences. Bootstrap values are indicated at branch points based on 1000 iterations. \textit{Streptococcus mitis} NCTC 12261\textsuperscript{T} was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

**Fig. 3.** Neighbour-joining tree of \textit{Lactococcus formosensis} sp. nov. 516\textsuperscript{T} and other related lactococci based on \textit{rpoB} sequences. Bootstrap values are indicated at branch points based on 1000 iterations. \textit{S. mitis} NCTC 12261\textsuperscript{T} was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.
Fig. 4. Neighbour-joining tree of *Lactococcus formosensis* sp. nov. 516T and other related lactococci based on pheS sequences. Bootstrap values are indicated at branch points based on 1000 iterations. *S. mitis* NCTC 12261T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Microbial Identification System (version 6.0), according to the instructions of the Microbial Identification System (MIDI).

Purified peptidoglycans of strain 516T contained alanine, glutamic acid and lysine. These data indicated that the cell-wall peptidoglycan type of strain 516T is of the A3γ type (Schleifer

Table 1. Characteristics of strain 516T and type strains of species and subspecies of the genus *Lactococcus* with validly published names

<table>
<thead>
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<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>9</th>
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<th>12</th>
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<td>+c</td>
<td>+</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>NA</td>
<td>–d</td>
<td>NA</td>
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<td>Growth in 6% (w/v) NaCl</td>
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<td>–</td>
<td>+c</td>
<td>–c</td>
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<td>–</td>
<td>W</td>
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<td>+c</td>
<td>W</td>
<td>–c</td>
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<td>+c</td>
<td>+c</td>
<td>+c</td>
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<td>+d</td>
<td>–d</td>
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<td>+c</td>
<td>–c</td>
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<td>+c</td>
<td>+c</td>
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<td>+d</td>
<td>++d</td>
<td>d,b,d</td>
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<td>–c</td>
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<td>+d</td>
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<td>–</td>
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<td>–c</td>
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<td>–c</td>
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<td>+d</td>
<td>b,d</td>
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<tr>
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<td>W</td>
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<td>–d</td>
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<td>NA</td>
<td>++d</td>
<td>–d</td>
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<tr>
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<td>34.6</td>
<td>45.7</td>
<td>45.7</td>
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<td>32.6</td>
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<tr>
<td>C_{18:1ω7c}</td>
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<td>31.3</td>
<td>3.18 (Summed feature 10)^†</td>
<td>22.9</td>
<td>9.9</td>
<td>60.5</td>
<td>14.3</td>
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<td>–e</td>
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<td>18.2</td>
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<td>–c</td>
<td>–c</td>
<td>–c</td>
<td>–c</td>
<td>–f</td>
<td>3.2</td>
<td>58.4</td>
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<tr>
<td>C_{19:0} cyclo 10c</td>
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<td>–c</td>
<td>–c</td>
<td>12.5</td>
<td>31.5</td>
<td>–c</td>
<td>26.3</td>
<td>–c</td>
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<td>–e</td>
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<td>–c</td>
<td>–f</td>
<td>2.0</td>
<td>–c</td>
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*Data obtained from: a, the Japan Collection of Microorganisms, RIKEN BioResource Center; b, the study of Collins et al. (1983); c, Chen et al. (2013); d, Cai et al. (2011); e, Cho et al. (2008); f, Pérez et al. (2011).
†Summed feature 10: C_{18:1ω7c} unknown ECL 17.834.
‡Summed feature 7: unknown 18.846 and C_{19:0} cyclo 10c.
The complete fatty acid profile of strain 516\textsuperscript{T} is shown in Table S2. The fatty acid profile of Lactococcus garvieae NBRC 100934\textsuperscript{T}, previously reported by Cho et al. (2008), differed from the profile of strain 516\textsuperscript{T} (Table S2).

The data reported here indicate the independent status of the isolated strain in the genus Lactococcus. Based on the results of DNA–DNA hybridization, 16S rRNA, rpoA, rpoB and pheS gene sequence analyses, the species of the isolated strain is clearly separate from its closest phylogenetic neighbours. In addition, there are some phenotypic characteristics that clearly distinguish strain 516\textsuperscript{T} from Lactococcus garvieae NBRC 100934\textsuperscript{T} (Table 1). The type strain has the characteristics given in the description of the species and in Table 1 and Tables S1 and S2.

In conclusion, the isolated strain from yan-tsai-shin, 516\textsuperscript{T}, represents the type strain of a novel species of the genus Lactococcus, named Lactococcus formosensis sp. nov.

**Description of Lactococcus formosensis sp. nov.**

Lactococcus formosensis [for.mo. sen’sis. N.L. masc. adj. formosensis of or pertaining to Formosa (Taiwan)].

Cells are Gram-stain-positive, catalase-negative, coccoid or ovoid-shaped, facultatively anaerobic and grow well anaerobically on MRS agar at 37 °C. Utilizes D-glucose homofermentatively and does not produce gas from glucose. Produces L-lactic acid from glucose. Grows at 20–37 °C, but not at 10 °C or 45 °C. Grows in 6 % NaCl but not at pH 5.0. Acid is produced from ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetylgalactosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, trehalose, \(\beta\)-gentiobiose and glucosamine. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-xylene, L-xylene, adonitol, methyl \(\beta\)-xyloside, L-sorbitose, rhamnose, dulcitol, inositol, sorbitol, methyl 2-D-mannoside, methyl 2-D-glucoside, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-fxyose, D-tagatose, D-fucose, L-fucose, L-arabitol, 2-ketogluconate and 5-ketogluconate. The major fatty acids detected in strain 516\textsuperscript{T} are C\textsubscript{16:0}, C\textsubscript{19:0} cyclo \(\omega\)8c and summed feature 7. The cell-wall peptidoglycan type is A3\(\alpha\).

The type strain is 516\textsuperscript{T} (=NBRC 109475\textsuperscript{T}, =BCRC 80576\textsuperscript{T}), which has a DNA G+C content of 38.1 mol%.

**Acknowledgements**

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**References**


