**Lactococcus taiwanensis** sp. nov., a lactic acid bacterium isolated from fresh cummingcordia

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One coccoc strain, designated 0905C15⁰, was isolated from fresh cummingcordia, which is the main ingredient of pobuzihi (fermented cummingcordia), a traditional fermented food in Taiwan. 16S rRNA gene sequencing results showed that strain 0905C15⁰ had 98.22–98.82 % sequence similarity to that of the type strains of four *Lactococcus lactis* subspecies (*L. lactis* subsp. *lactis* BCRC 12312⁰, *L. lactis* subsp. *cremoris* BCRC 12586⁰, *L. lactis* subsp. *hordniae* BCRC 80474⁰ and *L. lactis* subsp. *tructae* BCRC 80475⁰). Comparison of two housekeeping genes, *recA* and *rpoB*, revealed that strain 0905C15⁰ was well separated from the reference strains of the genus *Lactococcus*. DNA–DNA hybridization studies indicated that strain 0905C15⁰ had low DNA relatedness to the four *Lactococcus lactis* subspecies (9.7–15.24 %). The DNA G+C content of strain 0905C15⁰ was 39.6 mol %. Based on the evidence, strain 0905C15⁰ represents a novel species of the genus *Lactococcus*, for which the name *Lactococcus taiwanensis* sp. nov. is proposed. The type strain is 0905C15⁰ (=NBRC 109049⁰ = BCRC 80460⁰).

Lactic acid bacteria (LAB) are widely distributed in traditional fermented foods in Taiwan. In our previous study, we isolated a novel species of the genus *Lactobacillus* named *Lactobacillus pobuzihi* sp. nov. from pobuzihi (fermented cummingcordia) (Chen et al., 2010). In order to obtain more information on LAB diversity in pobuzihi, isolation of LAB was performed and a total of 196 LAB strains were isolated (Chen et al., 2013). All isolates were identified based on their phenotypic and phylogenetic characteristics. Phylogenetic analyses, based on 16S rRNA gene sequences, initially placed strain 0905C15⁰ within the species *Lactococcus lactis*. A total of seven species within the genus *Lactococcus* and four subspecies within the species *Lactococcus lactis* are currently recognized (Cai et al., 2011; Cho et al., 2008; Collins et al., 1983; Garvie & Farrow, 1982; Latorre-Guzman et al., 1977; Pérez et al., 2011). In order to exactly identify strain 0905C15⁰, analyses of two housekeeping genes, *recA* and *rpoB* (Pérez et al., 2011), and phenotypic characteristics was performed. The purpose of the present study was to establish the taxonomic position of this bacterial strain.

Strain 0905C15⁰ was isolated following the procedure of Chen et al. (2006) using de Man, Rogosa and Sharpe agar (MRS; Difco Lactobacilli MRS Broth) at 30 °C for 48 h. In addition, two reference strains, *L. lactis* subsp. *lactis* BCRC 12312⁰ and *L. lactis* subsp. *cremoris* BCRC 12586⁰, were obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan).

Strain 0905C15⁰ is a Gram-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 and 2010). Tests of acid production from carbohydrates were performed using the API50CHL fermentation kit (bioMérieux) according to the manufacturer’s instructions.

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA* and *rpoB* gene sequences of strain 0905C15⁰ are AB699722, AB699723 and AB699724, respectively.

Six supplementary figures and a supplementary table 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 recA and rpoB were performed using primers recALac1F (5'-GCAGCCTTTATCGATGCTG-3'), recAIR (5'-GCAC-GACCACCAGG-3'), rpoBLac1F (5'-TACGGKAAACACC-GTA-3') and rpoBLac1R (5'-TCAARCCAWGTCCACGGG-3'), which were designed and reported previously by Pérez et al. (2011). PCR was carried out using a TaKaRa Ex Taq gene amplification PCR kit (Takara Bio) and a Gene Amp PCR System 9700 (Perkin Elmer) following the methods described by Chen et al. (2006) and Pérez et al. (2011). DNA sequencing was performed using an ABI 3730 DNA Analyser (Applied Biosystems). Sequence homologies were assessed by comparing the obtained sequences with those in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using BLAST.

All sequences were aligned using the 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 method (Saitou & Nei, 1987), with distances calculated according to Kimura's two-parameter model (Kimura, 1980). The MEGA 5.05 package (Tamura et al., 2011) 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. 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 1463 nt), recA (approximately 347 nt) and rpoB (approximately 465 nt) genes were determined. Phylogenetic analysis of the 16S rRNA gene sequences obtained in this study and from GenBank indicated that strain 0905C15T belonged to the genus Lactococcus. Strain 0905C15T formed a monophyletic cluster with four type subspecies of Lactococcus lactis, supported by a bootstrap value of 100 % (Fig. 1).

When comparing the recA gene sequences with those held in the GenBank database, strain 0905C15T showed 98.18 % similarity to L. lactis subsp. lactis BCRC 12312T, 98.27 % similarity to L. lactis subsp. hordniae BCRC 80474T, 90.71 % similarity to L. lactis subsp. tructae BCRC 80475T and 90.41 % similarity to L. lactis subsp. cremoris BCRC 12586T. In agreement with these results, strain 0905C15T clustered with L. lactis subsp. lactis BCRC 12312T and L. lactis subsp. hordniae BCRC 80474T in the phylogenetic tree (Fig. 2).

Comparison of the rpoB gene with that of other Lactococcus lactis type strains was also performed. The results indicated that strain 0905C15T has 91.0 % sequence similarity to L. lactis subsp. cremoris BCRC 12586T, 90.7 % similarity to L. lactis subsp. tructae BCRC 80475T, 89.2 % similarity to L. lactis subsp. hordniae BCRC 80474T and 90.4 % similarity to L. lactis subsp. tructae BCRC 80475T.

Fig. 1. Neighbour-joining tree of L. taiwanensis sp. nov. 0905C15T and other related lactococci based on 16S rRNA sequences. Bootstrap values are indicated at branch points based on 1000 replications. GenBank accession numbers are given in parentheses. 16S rRNA sequences of L. garvieae NBRC 100934T were obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) database. Bacillus subtilis NCDO 1769T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.
L. lactis subsp. lactis BCRC 12312T and 88.9% to L. lactis subsp. hordniae BCRC 80474T. The phylogenetic analysis of the rpoB gene is shown in Fig. 3. The topology was also analysed using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971, 1977) methods. Bootstrap analysis was made with 1000 replicates. Similar results were obtained to that of the neighbour-joining method (Figs 1–3 and S1–S6 available in IJSEM Online).

Besides phylogenetic analyses, a number of phenotypic tests were performed. Strain 0905C15T showed homolactic acid fermentation and production of L-lactic acid and grew in a medium with 6% (w/v) NaCl. The tolerance to 6% NaCl clearly differentiated strain 0905C15T from L. lactis subsp. lactis BCRC 12312T, L. lactis subsp. cremoris BCRC 12586T, L. lactis subsp. hordniae BCRC 80474T and L. lactis subsp. tructae BCRC 80475T (Table 1). Strain 0905C15T grew better than the four strains representing the L. lactis subspecies at pH 5.0 (Table 1). Acid production from carbohydrates was assessed using the API 50CHL fermentation kit after 48 h of incubation; however, results differing from those for the four subspecies of L. lactis were observed (Table 1).

DNA G+C content of strain 0905C15T was 39.6 mol% which was different to those of the four subspecies of L. lactis (35.5–36.5 mol%) (Table 1). DNA–DNA hybridization experiments were performed using DNA derived from strain 0905C15T, L. lactis subsp. lactis BCRC 12312T (Schleifer et al., 1985), L. lactis subsp. cremoris BCRC 12586T (Schleifer et al., 1985), L. lactis subsp. hordniae BCRC 80474T (Schleifer et al., 1985) and L. lactis subsp. Lactococcus lactis subsp. cremoris BCRC 12586T, Lactococcus lactis subsp. hordniae BCRC 80474T and L. lactis subsp. tructae BCRC 80475T (Table 1).
Strain 0905C15<sup>T</sup> had low levels of DNA relatedness with *L. lactis* subsp. *lactis* BCRC 12312<sup>T</sup> (12.4 %), *L. lactis* subsp. cremoris BCRC 12586<sup>T</sup> (15.2 %), *L. lactis* subsp. hordniae BCRC 80474<sup>T</sup> (9.7 %) and *L. lactis* subsp. tructae BCRC 80475<sup>T</sup> (11.9 %). Values for DNA relatedness between strain 0905C15<sup>T</sup> and the four *L. lactis* strains ranged from 9.7 % to 15.24 %, indicating that strain 0905C15<sup>T</sup> is not part of the species *L. lactis* and supporting the result of the 16S rRNA gene sequence analyses.

Extraction and determination of cellular fatty acid profiles were performed by using the Sherlock Microbial Identification System (version 6.0), according to the instructions of the Microbial Identification System (MIDI). The major fatty acids detected in strain 0905C15<sup>T</sup> were C<sub>16:0</sub> (45.75 %), C<sub>18:1</sub> (18.26 %), C<sub>19:0</sub> cyclopropane 9,10 (10.31 %), C<sub>18:1</sub> (8.41 %), C<sub>18:0</sub> (4.47 %), C<sub>16:0</sub> DMA (1.04 %), C<sub>18:0</sub> 12-OH (0.68 %), C<sub>16:0</sub> aldehyde (0.61 %), C<sub>20:1</sub> (0.52 %), C<sub>16:1</sub> (0.50 %), C<sub>18:1</sub> (0.45 %), summed feature 10 (3.18 %), C<sub>18:1</sub> iso/unknown ECL 17.834) and summed feature 12 (1.35 %, C<sub>19:0</sub> iso/unknown ECL 18.622). In comparison to the fatty acid profiles of four subspecies of *L. lactis* which have been previously reported by Cho et al. (2008) and Pérez et al. (2011), the profile of strain 0905C15<sup>T</sup> is distinct (Table S1).

The data reported here indicate the independent status of the isolated strain in the genus *Lactococcus*. Based on the results of DNA–DNA hybridization, the isolated strain is clearly separate from its closest phylogenetic neighbours and there are some phenotypic characteristics that clearly distinguish strain 0905C15<sup>T</sup> from the type strains of the four subspecies of *L. lactis* (BCRC 12312<sup>T</sup>, BCRC 12586<sup>T</sup>, BCRC 80474<sup>T</sup> and BCRC 80475<sup>T</sup>; Table 1). The type strain has the characteristics given in the description of the species and in Table 1.

In conclusion, the strain isolated from pobuzihi is proposed to represent a novel species of the genus *Lactococcus*. The proposed name is *Lactococcus taiwanensis* with 0905C15<sup>T</sup> as the type strain.

**Description of *Lactococcus taiwanensis* sp. nov.**

*Lactococcus taiwanensis* (tai.wan.en’si.s N.L. masc. adj. taiwanensis of or belonging to Taiwan, referring to the geographical origin of the type strain).

Cells are Gram-positive, catalase-negative, cocccid or ovoid-shaped, facultatively anaerobic and grow well anaerobically on MRS agar at 30 °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 and at pH 5.0. Acid is produced from D-glucose, D-fructose, L-arabinose, trehalose, maltose, lactose, sucrose, N-acetylgalcosamine, galactose, D-mannose, ribose, mannotol, amylgladin, arbutin, aesculin, salicin, cellobiose, β-gentiobiose and gluconate. Acid is weakly produced from starch. Acid is not produced from melibiose, D-xylose, L-xylose, D-arabinose, erythritol, adonitol, methyl β-xylloside, L-sorbose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, inulin, melezitose, D-raffinose, glycosgen, xylitol, D-arabitol, L-arabitol, D-fucose, L-fucos, or D-turanose. The major cellular fatty acids are C<sub>16:0</sub>, C<sub>18:1</sub>ω9c and C<sub>19:0</sub> cyclopropane 9,10.

The type strain is 0905C15<sup>T</sup> (=NBRC 109049<sup>T</sup>=BCRC 80460<sup>T</sup>), isolated from fresh cummingcordia. The DNA G+C content of the type strain is 39.6 mol%.

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**References**

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