Lactobacillus capillatus sp. nov., a motile bacterium isolated from stinky tofu brine

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Three Gram-positive, catalase-negative, motile, rod-shaped bacteria were isolated from fermented stinky tofu brine. These strains, designated YIT 11306^T, YIT 11317 and YIT 11318, were discriminated from five isolates on the basis of randomly amplified polymorphic DNA profiles. They produced L-lactic acid as the main end product from glucose without gas formation, synthesized dextran from sucrose and hydrolysed aesculin. Ammonia was not produced from arginine. Comparative 16S rRNA gene sequence analysis demonstrated that the novel isolates were members of the genus *Lactobacillus*. Based on levels of 16S rRNA gene sequence similarity, the three novel strains were related most closely to the type strains of *Lactobacillus mali* (97.2%) and *Lactobacillus satsumensis* (96.8%). However, levels of DNA–DNA relatedness between the novel isolates and the type strains of *L. mali* and *L. satsumensis* were less than 10%. The phenotypic and genotypic data demonstrate that the three strains represent a single novel species of the genus *Lactobacillus*, for which the name *Lactobacillus capillatus* sp. nov. is proposed. The type strain is YIT 11306^T (=JCM 15044^T=BCRC 17811^T=DSM 19910^T).

Stinky tofu is a well-known and popular traditional fermented Chinese snack, and its manufacturing process differs from region to region. In Taiwan, stinky tofu is made by immersing tofu into fermented stinky brine, thus permeating the tofu with the odour of the brine. Stinky brine can be made entirely by mixing vegetables or may also include Chinese herbs, shrimp or fish to give a unique and strong odour. All of the raw materials decompose by natural fermentation, and the resulting liquid is the fermented brine used for production of stinky tofu.

During the course of a study of the biodiversity of lactic acid bacteria in stinky tofu brines, three *Lactobacillus*-like strains, designated YIT 11306^T, YIT 11317 and YIT 11318, could not be clearly placed within any recognized species of the genus by means of 16S rRNA gene sequence similarity. These strains are shown here to represent a single novel species of the genus *Lactobacillus*.

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YIT 11306^T, YIT 11317 and YIT 11318 are AB365976, AB365977 and AB365978, respectively.

Maximum-parsimony and maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences showing the relationship of strains YIT 11306^T, YIT 11317 and YIT 11318 to closely related *Lactobacillus* species are available as supplementary material with the online version of this paper.

Samples of fermented stinky brines were obtained in May 2005 from a factory for the manufacture of stinky tofu in Taipei County, Taiwan. Samples were taken aseptically, stored at 4 °C and analysed within 24 h. Stinky tofu brine samples were inoculated onto de Man-Rogosa-Sharpe (MRS; Difco) agar supplemented with 0.001% sodium azide and 0.001% cycloheximide and incubated anaerobically at 30 °C for 3 days. Cell counts of lactic acid bacteria were 4.2×10^3 – 1.2×10^6 c.f.u. ml⁻¹, and 168 isolates were collected for further analysis. The selected isolates were stored at –80 °C in nutrient broth (BD; Difco) containing 10% (v/v) DMSO. The present study describes the characterization of three strains (YIT 11306^T, YIT 11317 and YIT 11318) from the 168 isolates. *Lactobacillus agilis* YIT 0253^T, *Lactobacillus mali* YIT 0449^T, *Lactobacillus salivarius* subsp. *salivarius* YIT 0104^T and *Lactobacillus satsumensis* YIT 11287^T were obtained from the culture collection of the Yakult Central Institute (YIT; Tokyo, Japan) and were used as reference strains. The test strains were cultivated and maintained in MRS broth (pH 7.0) at 30 °C for 1 or 2 days, unless indicated otherwise.

Chromosomal DNA used as template for randomly amplified polymorphic DNA (RAPD) typing and 16S rRNA gene sequence amplification was prepared from the isolates according to the method of Zhu *et al.* (1993). Five isolates [ST-A9a^T (=YIT 11306^T), ST-A31a (=YIT 11317),

ST-A32a, ST-A33a (=YIT 11318) and ST-A39a] were initially screened by using RAPD fingerprinting as described by Akopyanz *et al.* (1992), with the following modification. Briefly, PCR was performed in 20 µl of a mixture containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 1.6 µM primer, 200 µM of each dNTP, 1.5 U *Taq* polymerase (Takara Bio Inc.) and 10 µg template DNA. Primers A (5'-CCGCAGCCAA-3'), B (5'-AACGCGCAAC-3') and C (5'-GCGGAAATAG-3') were used in the present study. The PCR cycling programme consisted of one cycle of 94 °C for 2 min, six cycles of 94 °C for 30 s, 36 °C for 1 min and 72 °C for 90 s, 30 cycles of 94 °C for 20 s, 36 °C for 30 s and 72 °C for 90 s, and finally one cycle of 72 °C for 3 min. Isolates ST-A31a and ST-A32a had identical RAPD profiles, as did isolates ST-A33a and ST-A39a (Fig. 1). Analysis of these profiles indicated that the five isolates could be discriminated into three strains, namely YIT 11306^T, YIT 11317 and YIT 11318.

Conditions for PCR amplification of the almost-complete 16S rRNA gene and subsequent DNA sequencing were as described by Miyake *et al.* (1998). Sequence assembly was performed by using the programs AUTOASSEMBLER version 2.1 (Applied Biosystems) and GENETYX-MAC version 13.0.1 (Software Development). The closest known relatives of the novel strains were determined according to the program FASTA. The 16S rRNA gene sequences (about 1460 bp) of strains YIT 11306^T, YIT 11317 and YIT 11318 and of those of their closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned by using the CLUSTAL_X program (version 1.82) (Thompson *et al.*, 1997). A phylogenetic tree was constructed with the neighbour-joining method (Saitou & Nei, 1987) by using the CLUSTAL_X program. The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates

(Felsenstein, 1985) and the tree topology was also confirmed with the maximum-parsimony (Fitch, 1971) and maximum-likelihood (Cavalli-Sforza & Edwards, 1967) methods, by using PHYLIP version 3.67 (Felsenstein, 2007). Levels of 16S rRNA gene sequence similarity between strain YIT 11306^T and strains YIT 11317 and YIT 11318 were 99.6 and 99.9%, respectively. Levels of similarity between strain YIT 11306^T and its nearest neighbours, *L. mali* YIT 0449^T and *L. satsumensis* YIT 11287^T, were 97.2 and 96.8%, respectively. Based on a neighbour-joining analysis, the novel strains did not belong to any existing phylogenetic groups and formed a distinct subcluster within the *L. mali* cluster, comprising *L. mali* and *L. satsumensis* (Fig. 2). Similar tree topologies were obtained by using the maximum-parsimony and maximum-likelihood methods (see Supplementary Figs S1 and S2, available in IJSEM Online).

For determination of levels of DNA–DNA relatedness and DNA G + C content, chromosomal DNA was extracted according to the method of Marmur (1961). DNA–DNA reassociation analyses were performed according to the microdilution well technique, by using photobiotin for DNA labelling (Ezaki *et al.*, 1989). Strain YIT 11318 was not included in the DNA–DNA hybridization studies because it showed very high 16S rRNA gene sequence similarity to strain YIT 11306^T. Reciprocal hybridization experiments were performed for each pair of strains at 40 °C for 15 min in the presence of 50 % formamide. Levels of DNA–DNA relatedness between strains YIT 11306^T and YIT 11317 were 74–88 %, indicating that these two strains belonged to the same taxon, whereas levels of DNA–DNA relatedness between strains YIT 11306^T and *L. agilis* YIT 0253^T, *L. mali* YIT 0449^T, *L. salivarius* subsp. *salivarius* YIT 0104^T and *L. satsumensis* YIT 11287^T were 6, 9, 8 and 9 %, respectively. These latter values were well below the 70 % cut-off value recommended for the distinction of separate bacterial species (Stackebrandt & Goebel, 1994). To assess DNA base compositions (G + C content), DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989) and then separated by HPLC. The DNA G + C contents of strains YIT 11306^T and YIT 11317 were 37.5 and 36.9 mol%, respectively.

Morphological, cultural and biochemical testing according to standard techniques was performed at 30 °C unless otherwise stated. Cell shape, cell size and Gram staining were determined by using cultures grown in MRS broth at 30 °C for 16 h. Motility was tested in MRS soft agar (0.15 %), and flagella on cells from 12-h cultures in MRS broth were observed under a transmission electron microscope (JEM-1200EX; JEOL) by negative staining with 2 % uranyl acetate. Transmission electron microscopy revealed the presence of peritrichous flagella (Fig. 3). Catalase activity was determined by using cells grown on MRS agar. Gas production from glucose was determined with a Durham tube in MRS broth. Production of dextran was assessed on MRS agar in which glucose was replaced with 2 % (w/v) sucrose. Growth at various temperatures

Fig. 1. Genetic relatedness among five novel *Lactobacillus* isolates by using RAPD–PCR analysis. Three primers with random sequences (A, B and C) were used. Lane M, 100-bp ladder DNA size marker (Seegene); lanes 1–5, isolates ST-A9a^T (YIT 11306^T), ST-A31a (YIT 11317), ST-A32a and ST-A33a (YIT 11318), and ST-A39a, respectively.

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains YIT 11306^T, YIT 11317 and YIT 11318 with closely related *Lactobacillus* species. The tree was constructed by using the neighbour-joining method on the basis of a comparison of approximately 1460 nt; *Lactobacillus delbrueckii* subsp. *bulgaricus* JCM 1002^T was used as an outgroup. Bootstrap values (%) based on 1000 replications are given at nodes. Bar, 1 % sequence divergence.

and pH, growth in the presence of NaCl, reduction of nitrate and production of ammonia from arginine were determined according to Barrow & Feltham (1993). Carbohydrate fermentation tests were carried out by using the API 50 CHL system (bioMérieux) according to the manufacturer's instructions. The isomers of lactic acid

formed from glucose were determined with a D/L-lactic acid enzymic kit (R-Biopharm AG).

Various phenotypic characteristics of the novel strains differed from those of *L. mali* (Carr & Davies, 1970; Kaneuchi *et al.*, 1988; Nonomura, 1983) and *L. satsumensis* (Endo & Okada, 2005), their phylogenetically closest relatives (Table 1). According to the data obtained, the three novel strains are genetically distinguishable from recognized species of lactic acid bacteria and thus represent a novel species, for which we suggest the name *Lactobacillus capillatus* sp. nov.

Description of *Lactobacillus capillatus* sp. nov.

Lactobacillus capillatus (ca.pil.la'tus. L. masc. adj. *capillatus* hairy, referring to the characteristic of having long, hairy flagella).

Cells are rod-shaped, 0.8–1.2 × 1.5–2 µm, and occur singly, in pairs or in short chains comprising three or four cells. Cells are Gram-positive, catalase-negative, motile by means of peritrichous flagella, non-spore-forming and facultatively anaerobic. After anaerobic growth at 30 °C for 48 h, colonies on MRS agar are 2–3 mm in diameter; they are white, opaque, smooth and circular with entire edges. In MRS broth, growth occurs at 15 °C but not at 45 °C. Growth occurs at pH 4.0 and pH 8.0. No growth occurs at 8 % NaCl. No gas is produced from glucose. Both L-lactic acid (80 %) and D-lactic acid (20 %) are produced as the end products from glucose. Ammonia is not produced from arginine. Nitrate is not reduced. Acid is produced from methyl β-xyloside, galactose, D-glucose, D-fructose, inositol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, melibiose, sucrose, xylitol and β-gentiobiose, but not from glycerol, erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, D-man-

Fig. 3. Transmission electron micrograph of peritrichous flagella of cells of strain YIT 11306^T grown in MRS broth at 30 °C for 12 h. Bar, 1 µm.

Table 1. Differential phenotypic characteristics between strains YIT 11306^T and YIT 11317 and genetically closely related lactobacilli

Taxa: 1, strain YIT 11306^T; 2, strain YIT 11317; 3, *L. mali*; 4, *L. satsumensis*. +, Positive; –, negative; (+), weakly positive; v, variable.

Characteristic	1	2	3	4
Acid production from:				
Ribose	–	–	v*	–†
Methyl β-xyloside	+	(+)	–‡	–‡
Galactose	+	+	+*	v†
D-Mannose	–	–	+‡	+‡
L-Sorbose	–	+	v*	+‡
Rhamnose	–	–	v*	+‡
Inositol	+	+	–*	–‡
Mannitol	–	+	+*	+†
Sorbitol	–	–	+‡	+‡
Salicin	+	+	–‡	+†
Cellobiose	+	–	+*	–†
Maltose	–	–	v*	+‡
Lactose	–	–	v*	–†
Melibiose	+	+	v*	–†
Trehalose	–	–	+*	+†
Inulin	–	–	+*	–‡
Melezitose	–	–	v*	v†
Raffinose	–	–	v*	–†
Xylitol	+	+	–‡	–‡
Turanose	–	–	–*	+‡
D-Tagatose	–	–	+*	+‡
L-Arabitol	–	+	–‡	–‡
Gluconate	–	–	–‡	v†
Growth at 45 °C	–	–	v*	+†
Catalase production	–	–	v*	–†
Isomer of lactic acid	L	L	DL§	L†
DNA G + C content (mol%)	37.5	36.9	32–33.7*	39–41†

*Data for reference species are from Kaneuchi *et al.* (1988).

†Data for reference species are from Endo & Okada (2005).

‡Data for reference species are from Rodas *et al.* (2006).

§Data for reference species are from Carr & Davies (1970).

nose, rhamnose, dulcitol, sorbitol, maltose, lactose, trehalose, inulin, melezitose, raffinose, starch, glycogen, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid production from L-arabinose, L-sorbose, mannitol, cellobiose and L-arabitol is strain-dependent. Aesculin is hydrolysed. Dextran is produced from sucrose. Cells contain *meso*-diaminopimelic acid in the peptidoglycan. The DNA G + C content of the type strain is 37.5 mol%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *L. mali* subgroup of lactobacilli.

The type strain, YIT 11306^T (=JCM 15044^T=BCRC 17811^T=DSM 19910^T), was isolated from fermented brine used for stinky tofu production in Taipei County, Taiwan, in 2005. YIT 11317 and YIT 11318, also isolated from stinky tofu brine, are additional strains of the species.

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