Lactobacillus satsumensis sp. nov., isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials

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Gram-positive, rod-shaped, motile lactic acid bacteria (strains NRIC 0603, NRIC 0604^T, NRIC 0605 and NRIC 0606) were isolated from shochu mashes using an enrichment culture approach. These strains clustered in the *Lactobacillus casei*–*Pediococcus* group and were closely related to *Lactobacillus nagelii* and *Lactobacillus mali* on the basis of 16S rRNA gene sequence similarity. Levels of DNA–DNA relatedness revealed genotypic separation of the four isolates from the above two species. The isolates are therefore considered to represent a novel species, for which the name *Lactobacillus satsumensis* is proposed. The type strain is NRIC 0604^T (=JCM 12392^T=DSM 16230^T).

During a study of lactic acid bacteria originating from plant material, 62 strains were isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice, sweet potato, barley and other starchy materials, together with *Saccharomyces cerevisiae* and *Aspergillus niger*. Fermented mashes contain 15–18% alcohol and they are kept at acidic pH (3.0–4.0) as a result of the production of citric acid by the mould during fermentation. The mash is therefore a harsh environment for lactic acid bacteria. After 16S rRNA gene sequence analysis of all the isolates, four strains (NRIC 0603, NRIC 0604^T, NRIC 0605 and NRIC 0606) underwent subsequent study because they clustered separately from recognized species of lactic acid bacteria. Levels of DNA–DNA relatedness also separated the strains from related species. This paper details the taxonomic study of the four strains and their proposed classification in a novel species, *Lactobacillus satsumensis* sp. nov.

The enrichment culture approach was employed for isolation of lactic acid bacteria from fermented mashes of shochu made from sweet potato that were collected at a shochu distillery in the South Kyushu district of Japan. The enrichment medium comprised 1.0% D-glucose, 1.0% yeast extract, 0.5% polypeptone, 0.2% sodium citrate, 10 p.p.m. sodium azide and 10 p.p.m. cycloheximide, and was adjusted to pH 5.0 with HCl. Fermented mashes were inoculated in the above medium and incubated at 30 °C. After incubation for 3 or 4 days at 30 °C, the cultured broth

was plated on MRS (Oxoid) agar containing 10 p.p.m. sodium azide and 10 p.p.m. cycloheximide and was then incubated. Colonies were picked on MRS agar and maintained on MRS agar containing 0.5% CaCO₃.

Chromosomal DNA was prepared from bacterial strains by the method of Zhu *et al.* (1993) and was then used as a template for 16S rRNA gene sequence amplification. Large fragments of the 16S rRNA gene sequence were amplified by PCR using two primers: 8F (5'-AGAGTTTGATCMTGGCTCAG-3', positions 8–27) and 15R (5'-AAGGAGGTGATCCARCCGCA-3', positions 1541–1522); position numbers were based on the *Escherichia coli* numbering system (GenBank accession no. V00348; Brosius *et al.*, 1981). The PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. They were sequenced using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 310; Applied Biosystems). The following seven primers were used: 8F, 930F (5'-GCACAAGCGGTGGAGCATGTGG-3', positions 933–954), 1400F (5'-TGTACACACCGCCCGT-3', positions 1391–1406), 520R (5'-ACCGCGGCTGCTGGC-3', positions 531–517), 800R (5'-CAGGACTACCAGGGTATCTAAT-3', positions 804–787), 1100R (5'-AGGGTTGCGCTCGTTG-3', positions 1115–1100) and 15R. The closest recognized relatives of the isolates were determined by performing database searches, and sequences of closely related species were retrieved from GenBank. Multiple alignments of the sequences were carried out with the program CLUSTAL X (version 1.18) (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated using the two-parameter method of Kimura (1980).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NRIC 0604^T is AB154519.

The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). The 16S rRNA gene sequence (positions 8–1541) was determined for NRIC 0604^T, and this was used to search for sequence similarity using DataBase. The highest sequence similarity to NRIC 0604^T was found with *Lactobacillus nagelii*, *Lactobacillus mali*, *Lactobacillus murinus* and *Pediococcus damnosus*, the values being 95.3, 95.0, 94.7 and 94.8 %, respectively; these species belong to the *Lactobacillus casei*–*Pediococcus* group (Fig. 1). Partial sequences (positions 8–531) of the 16S rRNA gene were determined for strains NRIC 0603, NRIC 0605 and NRIC 0606; these partial sequences were identical to one another and to that of NRIC 0604^T (positions 8–531). Therefore, we concluded that the four isolates belong to the same taxon and that this is located in the *Lactobacillus casei*–*Pediococcus* group.

Extraction and isolation of bacterial DNA were performed by the method of Marmur (1961) as modified by Ezaki *et al.* (1983). DNA–DNA hybridization was carried out by the microdilution well technique using photobiotin for labelling of DNA (Ezaki *et al.*, 1989). DNA base compositions (G + C content) of strains tested were determined by HPLC as described by Tamaoka & Komagata (1984). Strains NRIC 0603, NRIC 0604^T, NRIC 0605 and NRIC 0606 showed high levels of DNA–DNA relatedness (96–99 %) to one another. By contrast, NRIC 0604^T showed low levels of DNA–DNA relatedness to the type strains of *L. nagelii* (6 %) and *L. mali* (7 %). The DNA G + C content of the four isolates ranged from 39 to 41 mol%.

Cell shape, cell size and Gram staining (Hucker & Conn,

1923) were determined on cultures grown in MRS broth at 30 °C for 24 h. Motility was tested in MRS soft agar and flagella were stained by the method of Toda (1928). Catalase activity was determined on cells grown on MRS agar. Gas production from glucose was determined using a Durham tube in MRS broth. Nitrate reduction, acid formation from carbohydrates and production of dextran from sucrose were determined by the methods described by Tanasupawat *et al.* (1998). The effects of temperature and various initial pH levels were tested in MRS broth. Resistance to NaCl and ethanol was examined in MRS broth containing 5 % (w/v) NaCl or 10 % (v/v) ethanol after incubation for 5 days at 30 °C. Type of fermentation was determined enzymically using the F-kit ethanol (Roche Diagnostics) as described by Okada *et al.* (1991). The isomer type of lactic acid was analysed by HPLC as described by Manome *et al.* (1998). Preparation of cell walls and determination of peptidoglycans were carried out by the methods described by Komagata & Suzuki (1987), except that TLC was performed on cellulose sheets. Various phenotypic characteristics of the isolates were similar to those of *L. nagelii* (Edwards *et al.*, 2000) and *L. mali* (Carr & Davies, 1970; Nonomura, 1983; Kaneuchi *et al.*, 1988), which are the phylogenetically closest related species (Table 1). In addition to the strains tested, *L. nagelii* and *L. mali* are motile, have *meso*-diaminopimelic acid in the cell wall and produce dextran from sucrose. These characteristics are unique in the genus *Lactobacillus*. The strains investigated cannot be distinguished from *L. nagelii* and *L. mali* based on their phenotypic characteristics, and levels of DNA–DNA relatedness were therefore used to provide accurate classification.

Based on the data provided, the four isolates are genetically distinguishable from recognized species of lactic acid bacteria, and thus represent a novel species, for which the name *Lactobacillus satsumensis* sp. nov. is proposed.

Fig. 1. Phylogenetic relationships of isolates in the *Lactobacillus casei*–*Pediococcus* group based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method, and approximately 1440 nt were used for comparison. *Escherichia coli* was used as an outgroup.

Table 1. Characteristics of *Lactobacillus satsumensis* sp. nov. and genetically closely related lactobacilli

Data for reference species are from Kaneuchi *et al.* (1988) (*L. mali*) and Edwards *et al.* (2000) (*L. nagelii*); motility and the presence of *meso*-diaminopimelic acid in the cell wall of *L. nagelii* NRIC 0559^T were determined in the present study. +, Positive; –, negative; V, variable. All three species are motile, produce acid from D-fructose, D-trehalose and D-salicin and have *meso*-diaminopimelic acid in the cell wall. All three species are negative for acid production from D-ribose, D-xylose and lactose.

Characteristic	<i>L. satsumensis</i> sp. nov.	<i>L. mali</i>	<i>L. nagelii</i>
Catalase production	–	V	–
Acid from D-cellobiose	–	V	+
Growth in MRS broth containing 5 % NaCl	+	V	+

Fig. 2. Peritrichous flagella of *Lactobacillus satsumensis* sp. nov. NRIC 0604^T grown on an MRS slant at 30 °C for 16 h.

Description of *Lactobacillus satsumensis* sp. nov.

Lactobacillus satsumensis [sat.su.men'sis. N.L. masc. adj. *satsumensis* pertaining to Satsuma (old name for the southern part of Kyushu in Japan), from where the type strain was isolated].

Cells are Gram-positive rods measuring 0.6–0.8 × 1.0–1.5 µm. Cells occur singly or in pairs, and are motile with peritrichous flagella (Fig. 2). They are facultatively anaerobic and catalase-negative. Colonies on MRS agar are white, smooth and approximately 2 mm in diameter after incubation for 2 days. They are homofermentative and produce L-lactic acid as a main end-product from D-glucose. Nitrate is not reduced. Acid is produced from D-fructose, D-mannose, sucrose, D-salicin, D-trehalose and D-mannitol, but not from L-arabinose, D-ribose, D-xylose, D-cellobiose, lactose, melibiose, D-raffinose or starch. Some strains produce acid from D-gluconate, D-galactose and D-melezitose. Dextran is formed from sucrose. Cells grow at 15 and 45 °C but not at 4 or 50 °C. Growth is observed in MRS broth containing 5 % (w/v) NaCl and at pH 3.5 in MRS broth. No growth is observed in MRS broth containing 10 % (v/v) ethanol. Cells contain *meso*-diaminopimelic acid in their peptidoglycan. The DNA G+C content ranges from 39 to 41 mol% (type strain 40 mol%).

The type strain is NRIC 0604^T (=JCM 12392^T=DSM 16230^T). All strains were isolated from shochu mashes collected at a shochu distillery in the South Kyushu district of Japan.

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