Lactobacillus sucicola sp. nov., a motile lactic acid bacterium isolated from oak tree (*Quercus* sp.) sap

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Three strains of rod-shaped and motile lactic acid bacteria, NRIC 0736^T, NRIC 0735 and NRIC 0737 were isolated from sap obtained from two different oak trees (*Quercus* sp.) on Awashima Island, Kagawa Prefecture, Japan. The three strains showed nearly identical 16S rRNA gene sequences (>99.7% sequence similarity). The novel strains showed low 16S rRNA gene sequence similarities to recognized species of lactic acid bacteria. High gene sequence similarities were found between strain NRIC 0736^T and *Lactobacillus satsumensis* NRIC 0604^T (96.3%), *Lactobacillus mali* DSM 20444^T (95.3%) and *Lactobacillus vini* DSM 20605^T (95.3%). DNA–DNA relatedness values revealed the genotypic separation of the three novel isolates from these recognized species. It is concluded that the three new isolates represent a novel species, for which the name *Lactobacillus sucicola* sp. nov. is proposed. The type strain is NRIC 0736^T (=JCM 15457^T=DSM 21376^T).

During a study of the diversity of lactic acid bacteria (LAB) on plant material, 31 strains were isolated from sap obtained from two different oak trees (*Quercus* sp.), A and B, from Awashima Island, Kagawa Prefecture, Japan, in July 2006. Beetles, hornets and other insects are known to suck the sap from these trees. After 16S rRNA gene sequence analysis of all the isolates, three strains, NRIC 0736^T and NRIC 0735 (from tree A) and NRIC 0737 (from tree B) were chosen for subsequent study because they showed low 16S rRNA gene sequence similarities with known LAB species (<97%). Moreover, levels of DNA–DNA relatedness also separated the novel strains from known LAB. Based on the results of phenotypic and genotypic analyses in this study, the three new strains were found to represent a novel species of LAB.

The enrichment culture approach was employed to isolate LAB from the oak tree sap. MRS broth (Oxoid) was used as the isolation medium. This broth contained inhibitors, 10 p.p.m. sodium azide and 10 p.p.m. cycloheximide, to inhibit the growth of aerobic bacteria and fungi. The oak tree sap was added to MRS broth and incubated at 30 °C. After incubation for 3–4 days, the broth was plated out on MRS agar (Oxoid) containing 0.5% CaCO₃ and the previously mentioned inhibitors. The plates were incubated

at 30 °C. Colonies with a clear zone, identified as acid-producing bacteria, were randomly selected from the plates. These were then maintained on MRS agar containing CaCO₃.

Chromosomal DNA was prepared from the novel strains using the method of Zhu *et al.* (1993). The DNA was then used as a template for 16S rRNA gene sequence amplification. The amplification was performed using two primers: 8F (5'-AGAGTTTGATCMTGGCTCAG-3', positions 8–27) and 15R (5'-AAGGAGGTGATCCARCCGCA-3', positions 1541–1522). The position numbers were based on the *Escherichia coli* numbering system (GenBank accession no. V00348; Brosius *et al.*, 1981). The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The products were sequenced using a Big Dye Terminator v.1.1 cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 310 Genetic Analyzer; ABI PRISM). The seven oligonucleotides 8F, 930F (5'-GC-ACAAGCGGTGGAGCATGTGG-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 were used as primers for sequencing 16S rRNA gene fragments. The closest recognized relatives of the novel isolates were determined by searching 16S rRNA gene sequences obtained from the EMBL/GenBank/DDBJ databases. These sequences were aligned using CLUSTAL_X (version 1.18) (Thompson *et al.*, 1997). A distance matrix was obtained by the two-parameter method of Kimura (1980). The neighbour-joining method

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains NRIC 0735, NRIC 0736^T and NRIC 0737 are AB458681, AB433982 and AB458682, respectively.

A micrograph of a cell of *Lactobacillus sucicola* NRIC 0736^T grown on an MRS slant at 30 °C for 15 h showing peritrichous flagella is available with the online version of this paper.

was used to construct a phylogenetic tree (Saitou & Nei, 1987). The robustness of the individual branches of the tree was established by bootstrapping with 1000 replicates (Felsenstein, 1985).

The 16S rRNA gene sequences (position 8–1541) of strains NRIC 0736^T, NRIC 0735 and NRIC 0737 were determined and compared with each other. Strain NRIC 0736^T showed 99.7 % and 99.7 % gene sequence similarities with strains NRIC 0735 and NRIC 0737, respectively. The sequence of strain NRIC 0736^T was compared with those of recognized species. Strain NRIC 0736^T showed high gene sequence similarities to *Lactobacillus satsumensis* NRIC 0644^T (96.6 %), *Lactobacillus mali* DSM 20444^T (95.3 %) and *Lactobacillus vini* DSM 20605^T (95.3 %). It was therefore concluded that the three novel isolates belonged to the same taxon and were located in the *Lactobacillus casei*–*Pediococcus* group (Fig. 1) (Collins *et al.*, 1991).

Extraction and isolation of bacterial DNA was performed according to the method of Marmur (1961) as modified by Ezaki *et al.* (1983). DNA–DNA hybridization was carried out with the fluorometric hybridization technique in microdilution wells with labelling of DNA with photobiotin (Ezaki *et al.*, 1989). The DNA G + C contents of the strains were determined by HPLC as described by Tamaoka & Komagata (1984).

Strains NRIC 0736^T, NRIC 0735 and NRIC 0737 showed high levels of DNA–DNA relatedness (88–100 %) with one another. Therefore, it was concluded that the new isolates belong to the same taxon. In contrast, strain NRIC 0736^T showed low levels of DNA–DNA relatedness to the type strains of *L. satsumensis* (11 %) and *L. mali* (11 %). The

DNA G + C contents of the three novel strains ranged from 37 to 38 mol% (Table 1).

Cell morphology and Gram staining (Hucker & Conn, 1923) were examined using cells grown in GYP broth at 30 °C for 24 h. GYP broth comprised (l⁻¹ distilled water): 10 g D-glucose, 10 g yeast extract, 5.0 g peptone, 2.0 g C₂H₃O₂Na . 3H₂O, 0.5 g Tween 80, 200 mg MgSO₄ . 7H₂O, 10 mg MnSO₄ . 4H₂O, 10 mg FeSO₄ . 7H₂O and 10 mg NaCl; pH was adjusted to pH 6.8 with NaOH. Motility was tested in GYP soft agar and flagella were stained by the method of Toda (1928). Catalase activity was determined by the addition of 3 % (w/v) H₂O₂ to the fresh cells grown on GYP agar. Gas production from glucose was determined using a Durham tube in GYP broth. The type of fermentation was determined using an F-kit ethanol (Roche Diagnostic) as described by Okada *et al.* (1991). The isomer of lactic acid was determined by HPLC as described by Manome *et al.* (1998). Acid formation from carbohydrates was assessed by the use of a basal medium of GYP broth with glucose omitted. The acid produced in 2 ml broth was titrated with 0.1 M NaOH. The effect of temperature (10, 15, 30, 37, 45 and 50 °C) on growth was tested by using GYP broth. Tolerance to NaCl was examined in GYP containing 5 % (w/v) NaCl after incubation for 5 days at 30 °C. The preparation of cell walls and the determination of peptidoglycan types were carried out according to Komagata & Suzuki (1987).

Production of dextran from sucrose was investigated by culturing on SYP plates. SYP medium comprised (l⁻¹ distilled water): 20 g sucrose, 10 g yeast extract, 10 g peptone, 5.0 g C₂H₃O₂Na . 3H₂O, 0.5 g Tween 80, 200 mg

Fig. 1. Phylogenetic relationships of *Lactobacillus sucicola* NRIC 0736^T and species in the *Lactobacillus casei*–*Pediococcus* group based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method and approximately 1400 nt were used for comparison. *Oenococcus oeni* JCM 6125^T was used as an outgroup. Bar, 1 % sequence divergence.

Table 1. DNA G+C contents and DNA–DNA relatedness between *Lactobacillus sucicola* sp. nov. and phylogenetically closely related species of the genus *Lactobacillus*

Strain	DNA G+C content (mol%)	Hybridization (%) with DNA from:				
		1	2	3	4	5
1. <i>Lactobacillus sucicola</i> NRIC 0735	37	(100)				
2. <i>Lactobacillus sucicola</i> NRIC 0736 ^T	38	89	(100)			
3. <i>Lactobacillus sucicola</i> NRIC 0737	38	88	100	(100)		
4. <i>Lactobacillus satsumensis</i> NRIC 0644 ^T	40	8	11	11	(100)	
5. <i>Lactobacillus mali</i> NRIC 1692 ^T	33	9	11	11	11	(100)

MgSO₄·7H₂O, 10 mg MnSO₄·4H₂O, 10 mg FeSO₄·7H₂O, 10 mg NaCl and 12 g agar; the pH was adjusted to pH 6.8 with NaOH. The three novel strains were cultured on SYP agar plates at 30 °C. After 3 days incubation, all of the novel strains produced a viscid substance around the colonies. The viscid substance was scraped from the plate and was suspended in 0.5 ml water in a 2.0 ml microtube. After the addition of 1.0 ml 99% ethanol, the microtubes were kept at 4 °C for 30 min. The suspension was then centrifuged at 6000 g for 3 min, the supernatant was removed and the precipitate was dried. Approximately 1 mg dried material was hydrolysed with 1.0 ml 6M HCl in a vacuum hydrolysis tube at 100 °C for 6 h. After cooling, the hydrolysate was concentrated to dryness using a rotary evaporator. The dried material was dissolved in 30 µl water and a 3 µl sample was then applied on the base line of a cellulose TLC plate (Merck). The TLC plate was developed with a solvent system of iso-propanol/pyridine/acetic acid/water (8:8:1:4, v/v). Development took approximately 3 h or more. Spots were visualized with AgNO₃ reagent. One spot appeared. Glucose was determined to be present in this spot; other sugars were not detected.

The characteristics of the novel isolates are shown in Table 2 (details are also given in the species description). Some phenotypic characteristics of the novel isolates were

Table 2. Differential characteristics of *Lactobacillus sucicola* sp. nov. and closely related lactobacilli

Strains: 1, NRIC 0735; 2, NRIC 0736^T; 3, NRIC 0737; 4, *L. mali* (unless indicated, data from Kaneuchi *et al.*, 1988); 5, *L. satsumensis* (Endo & Okada, 2005). +, Positive; –, negative; v, variable.

Characteristic	1	2	3	4	5
Motility	+	+	+	+	+
Catalase production	–	–	–	v	–
Acid from:					
Cellobiose	–	–	–	v	–
D-Galactose	+	+	+	–	+
Maltose	–	+	+	–	–
Melibiose	+	+	+	–	–
Raffinose	–	+	–	–	–
Growth in MRS broth containing 5% NaCl	–	–	–	v	+

different from those of *L. satsumensis* (Endo & Okada, 2005) and *L. mali* (Carr & Davies, 1970; Nonomura, 1983; Kaneuchi *et al.*, 1988). Some phenotypic characteristics of the novel isolates were similar to those of *L. satsumensis* and *L. mali*, for example the novel strains were motile with peritrichous flagella (See Supplementary Fig. S1 in IJSEM Online).

On the basis of the results of this study, the three new isolates are regarded as representing a novel species in the genus *Lactobacillus*, for which the name *Lactobacillus sucicola* sp. nov. is proposed.

Description of *Lactobacillus sucicola* sp. nov.

Lactobacillus sucicola [su'ci.co'la. L. n. *sucus* sap; L. suff. *-cola* (from L. n. *incola*) inhabitant, dweller; N.L. n. *sucicola* a sap-dweller].

Cells are Gram-positive rods measuring 1.0 × 2.0–3.0 µm. Cells occur singly or in pairs and are motile by means of peritrichous flagella. Facultatively anaerobic and catalase-negative. Colonies are white, smooth and 2 mm diameter on MRS agar plates incubated at 30 °C for 3–4 days. Homofermentative. No gas is produced from D-glucose. L-Lactic acid is produced from glucose as the main end product. Acid is produced from D-glucose, D-fructose, D-galactose, D-mannose, melibiose, sucrose, D-salicin, trehalose, D-mannitol and sorbitol, but not from L-arabinose, D-ribose, D-xylose, gluconate, rhamnose, cellobiose, lactose, melezitose or starch. Acid production from maltose and raffinose is variable between strains. Dextran is produced from sucrose. Cells grow at 15 and 45 °C, but not at 10 or 50 °C. Growth is not observed in GYP broth containing 5% (w/v) NaCl. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The DNA G+C content ranges from 37 to 38 mol% (type strain, 38 mol%).

The type strain, NRIC 0736^T (=JCM 15457^T=DSM 21376^T), was isolated from the sap of an oak (*Quercus* sp.) from Awashima Island, Kagawa Prefecture, Japan. Additional strains were also isolated from the oak sap.

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