Lactobacillus nasuensis sp. nov., a lactic acid bacterium isolated from silage, and emended description of the genus *Lactobacillus*

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Two strains of lactic acid bacteria, designated SU 18^T and SU 83, were isolated from silage prepared with Sudan grass [*Sorghum sudanense* (Piper) Stapf.]. The isolates were Gram-stain-positive, catalase-negative, facultatively anaerobic rods that did not produce gas from glucose. The isolates exhibited $\geq 93.5\%$ DNA–DNA relatedness to each other and shared the same phenotypic characteristics, which indicated that they belonged to a single species. The DNA G + C content was 58.5–59.2 mol%. On the basis of 16S rRNA gene sequence analysis, the isolates were placed in the genus *Lactobacillus*. Their closest phylogenetic neighbours were *Lactobacillus manihotivorans* JCM 12514^T and *Lactobacillus camelliae* JCM 13995^T (95.9 and 96.8% 16S rRNA gene sequence similarity, respectively, with strain SU 18^T). Ribotyping revealed that strain SU 18^T was well separated from *L. manihotivorans* JCM 12514^T and *L. camelliae* JCM 13995^T. Strain SU 18^T exhibited $\leq 23.7\%$ DNA–DNA relatedness with its closest phylogenetic neighbours. The isolates represent a novel species in the genus *Lactobacillus*, for which the name *Lactobacillus nasuensis* sp. nov. is proposed. The type strain is SU 18^T (=JCM 17158^T =CGMCC 1.10801^T). The description of the genus *Lactobacillus* is also amended.

Silage is now the most common preserved ruminant feed. Epiphytic lactic acid bacteria (LAB), naturally present on forage crops, are responsible for silage fermentation and also influence silage quality (Cai, 1999). Species of the genus *Lactobacillus* are major components of the microbial flora in various types of forage crops and silages and they usually play an important role in lactic acid production and pH reduction during silage fermentation (Cai *et al.*, 1998; Pang *et al.*, 2011). Sudan grass [*Sorghum sudanense* (Piper) Stapf.] is a very popular forage crop that is widely used for silage production in many countries, including Japan.

In the present study, members of the genus *Lactobacillus* were isolated from Sudan grass silage and examined by phenotypic analysis, 16S rRNA gene sequence analysis, ribotyping and DNA–DNA hybridization. Sudan grass at the milk stage was obtained from an experimental field at the National Institute of Livestock and Grassland Science, Nasushiobara, Tochigi, Japan. Silage was prepared using a

small-scale fermentation system (Cai *et al.*, 1998) and was stored for 45 days. Silage samples (10 g) were blended with 90 ml of sterilized distilled water and serial dilutions were incubated on lactobacilli MRS agar (Difco). Two strains were isolated from two samples incubated at 30 °C for 48 h in an anaerobic box (TE-HER Hard Anaerobox ANX-1; Hirosawa). Each strain was purified by streaking twice on MRS agar. The isolates were maintained on MRS agar at 30 °C for 48 h and stored in nutrient broth (Difco)/DMSO (9:1) at –80 °C. *Lactobacillus manihotivorans* JCM 12514^T and *Lactobacillus camelliae* JCM 13995^T were obtained from the Japan Collection of Microorganisms at the Institute of Physical and Chemical Research, Wako, Saitama, Japan.

Morphological and Gram-staining characteristics were examined after incubation for 48 h on MRS agar. Catalase activity and gas production from glucose were determined by the methods of Kozaki *et al.* (1992). Growth in MRS broth was observed after incubation at 5, 10 and 15 °C for 14 days and 40 and 45 °C for 7 days, with 3.0 and 6.5 % NaCl for 7 days and at pH 3.0–6.0 (at intervals of 0.5 pH units), 7.0 and 8.0 at 30 °C for 7 days. Carbohydrate fermentation tests and enzymic activities were determined using the API 50 CH and API ZYM systems (bioMérieux),

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SU 18^T and SU 83 are AB608051 and AB608052, respectively.

according to the manufacturer's instructions. The optical activity and quantity of lactic acid was determined using an enzymic bioanalysis and food analysis kit (R-Biopharm Aktiengesellschaft), in accordance with the manufacturer's instructions.

For 16S rRNA gene sequence analysis, cells were grown for 8 h in MRS broth at 30 °C and DNA was extracted and purified as described by Cai (1999). Amplification of the 16S rRNA gene was carried out using a Takara Taq PCR kit (Takara Shuzo Co) and a GeneAmp PCR System 9700 (PE Applied Biosystems) as described by Cai (1999). Sequencing was performed twice on both strands by the dideoxy method of Sanger *et al.* (1977), using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and a model 310A automated sequencing system (Applied Biosystems). The sequence information was imported into DNASTAR software for assembly. The 16S rRNA gene sequences of strains SU 18^T and SU 83 and those of related strains in public databases were compared using BLAST and aligned using CLUSTAL W (Hitachi Software Engineering Co). Tree topologies were evaluated by bootstrap analysis using MEGA version 5.0 (Tamura *et al.*, 2011) based on 1000 random resamplings (Ben-Dov *et al.*, 2006). Nucleotide substitution rates (K_{nuc}) were calculated (Kimura & Ota, 1972) and phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-likelihood (Tamura *et al.*, 2011) algorithms using MEGA 5.0 and the Kimura two-parameter model. *Bacillus subtilis* NCDO 1769^T was used as an outgroup.

Ribotyping was performed using *EcoRI* as the restriction enzyme and the automated ribotyping device RiboPrinter (Qualicon), in accordance with the manufacturer's instructions. Ribopatterns were analysed by using BioNumerics version 2.5 (Applied Maths) and compared by Pearson similarity coefficient analysis and the unweighted pair group method with arithmetic mean algorithm (UPGMA) (Cai *et al.*, 2011).

To determine DNA base composition and DNA–DNA relatedness, DNA was extracted from cells grown in MRS broth for 8 h at 30 °C and purified by the procedure of Saito & Miura (1963). DNA base composition was determined by HPLC following enzymic digestion of DNA to deoxyribonucleosides, according to the method of Tamaoka & Komagata (1984). An equimolar mixture of the four deoxyribonucleotides in a GC kit (Yamasa Shoyu Co) was used as the quantitative standard. DNA–DNA relatedness was determined by the method of Ezaki *et al.* (1989) using photobiotin and microplates.

Strains SU 18^T and SU 83 were Gram-stain-positive, non-spore-forming and non-motile. Cells were short, slender rods measuring 2.9–3.0 × 8.7–9.0 µm in MRS broth at 30 °C and occurred singly or in pairs. After incubation on MRS agar for 48 h, colonies were white, opaque, circular, convex and smooth, with a diameter of 7.0–8.0 mm.

Table 1. Phenotypic properties of strains SU 18^T and SU 83 and their closest phylogenetic neighbours

Strains: 1, *Lactobacillus nasuensis* sp. nov. SU 18^T; 2, *Lactobacillus nasuensis* sp. nov. SU 83; 3, *L. manihotivorans* JCM 12514^T; 4, *L. camelliae* JCM 13995^T. All data were taken from this study. All strains are catalase-negative rods that do not produce gas from glucose, are able to grow at 40 °C, with 3.0% NaCl and at pH 4.5–8.0, and produce acid from galactose, D-glucose, D-fructose and N-acetylglucosamine. None of the strains are able to grow at 5 or 45 °C or at pH 3.0 or produce acid from glycerol, erythritol, D-arabinose, L-sorbose, L-xylose, adonitol, methyl β-D-xyloside, dulcitol, inositol, sorbitol, methyl α-D-mannoside, inulin, xylitol, D-lyxose, D- or L-fucose, melezitose, D- or L-arabitol, gluconate, turanose, D-tagatose or 5-ketogluconate. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4
Growth at/with:				
10 °C	w	w	–	–
6.0% NaCl	w	w	–	–
pH 3.5	–	–	w	w
pH 4.0	w	w	+	+
Acid from:				
L-Arabinose	w	w	–	–
Ribose	+	+	–	–
D-Xylose	w	w	–	+
D-Mannose	–	–	+	+
Rhamnose	–	–	w	–
Mannitol	–	–	–	w
Methyl α-D-glucoside	–	–	+	–
Amygdalin	–	–	+	+
Arbutin	–	–	+	+
Aesculin	–	–	+	+
Salicin	–	–	+	+
Cellobiose	–	–	+	+
Maltose	w	w	+	+
Lactose	–	–	+	–
Melibiose	–	–	+	–
Sucrose	–	–	+	–
Trehalose	–	–	+	–
Raffinose	–	–	+	–
Starch	–	–	+	–
Glycogen	–	–	+	–
β-Gentiobiose	–	–	+	+
2-Ketogluconate	w	w	–	–
Enzymic activity				
α-Galactosidase	w	w	–	–
β-Galactosidase	+	+	–	+
α-Fucosidase	–	–	–	+

Both isolates were catalase-negative and did not produce gas from glucose, which indicated that they were homo-fermentative. The isolates grew well under aerobic and strictly anaerobic conditions with liquid and solid MRS media. Strains SU 18^T and SU 83 and the two reference strains grew well at 40 °C, with 3.0% NaCl and at pH 4.5–8.0, but did not grow at 5 or 45 °C or at pH 3.0. The

Fig. 1. Neighbour-joining phylogenetic tree showing the positions of strains SU 18^T and SU 83 in the genus *Lactobacillus*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. *Bacillus subtilis* NCDO 1769^T was used as an outgroup. Bar, 1 % sequence divergence.

optimal temperature for growth was approximately 30 °C. All strains produced acid from galactose, D-glucose, D-fructose and N-acetylglucosamine, but failed to produce acid from glycerol, erythritol, D-arabinose, L-sorbose, L-xylose, adonitol, methyl β-D-xyloside, dulcitol, inositol, sorbitol, melezitose, methyl α-D-mannoside, inulin, xylitol, D-lyxose, D- and L-fucose, D- and L-arabitol, gluconate, turanose, D-tagatose and 5-ketogluconate. As shown in Table 1, strains SU 18^T and SU 83 exhibited similar phenotypic characteristics. The isolates were clearly different from *L. manihotivorans* JCM 12514^T and *L. camelliae* JCM 13995^T in the fermentation of ribose, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose and β-gentiobiose. Strains SU 18^T and SU 83 were

weakly positive for fermentation of L-arabinose, D-xylose, maltose and 2-ketogluconate and produced α- and β-galactosidases, but did not produce α-fucosidase, which allowed them to be easily distinguished from the reference strains.

The 16S rRNA gene sequences obtained from strains SU 18^T and SU 83 comprised more than 1500 base pairs. 16S rRNA gene sequence similarity between strains SU 18^T and SU 83 was 100 %. Phylogenetic trees were reconstructed from evolutionary distances by the neighbour-joining (Fig. 1), minimum-evolution and maximum-likelihood (Fig. S1, available in IJSEM Online) methods, respectively. Strains SU 18^T and SU 83 were members of the genus *Lactobacillus*.

Fig. 2. Dendrogram illustrating the relatedness of the ribotyping patterns of strain SU 18^T and its closest phylogenetic neighbours in the genus *Lactobacillus*. The dendrogram was analysed using Pearson similarity coefficient analysis and UPGMA.

Strain SU 18^T was most closely related to *L. manihotivorans* JCM 12514^T and *L. camelliae* JCM 13995^T (95.9 and 96.8 % 16S rRNA gene sequence similarity, respectively). The isolates and their closest neighbours formed a distinct cluster within the genus *Lactobacillus*. 16S rRNA gene sequence similarity between strain SU 18^T and the other type strains tested was <95.8 %.

Ribotyping was applied to investigate further the relationships between strain SU 18^T and *L. manihotivorans* JCM 12514^T and *L. camelliae* JCM 13995^T. Strain SU 18^T exhibited a pattern that was quite different from the reference strains and formed a distinct and well-separated branch (Pearson correlation <30 %) (Fig. 2).

DNA base composition and DNA–DNA hybridization data are shown in Table 2. The DNA G + C contents of strains SU 18^T and SU 83 were 59.2 and 58.5 mol%, respectively, and of *L. manihotivorans* JCM 12514^T and *L. camelliae* JCM 13995^T were 48.4 and 56.0 mol%. DNA–DNA relatedness between the isolates was ≥93.5 % and between strain SU 18^T and *L. manihotivorans* JCM 12514^T and *L. camelliae* JCM 13995^T was ≤23.7 %.

Species of the genus *Lactobacillus* are widely distributed on various plant surfaces and in silage and animal intestines (Cai *et al.*, 1998; Ennahar *et al.*, 2003; Spicher & Schröder, 1978, 1980). There have been several reports of lactobacilli composing the major microbial population of forage crops and silage, where they may contribute to silage fermentation. Some silage-associated lactobacilli have been characterized by phenotypic features and 16S rRNA gene sequences and have been described as novel species: for example, *L. paraplantarum*, *L. brevis*, *L. buchneri*, *L. acidophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L.*

pentosus (Cai *et al.*, 1998; Cai, 1999; Ennahar *et al.*, 2003; Moon, 1984; Pang *et al.*, 2011; Tannock, 1999).

On the basis of phenotypic and genotypic characteristics, it is concluded that the isolates represent a novel species of the genus *Lactobacillus*, for which we propose the name *Lactobacillus nasuensis* sp. nov. The G + C content of the genus *Lactobacillus* ranges from 32 to 55 mol% (Liu & Dong, 2002); however, the G + C contents of strains SU 18^T and SU 83 were 58.5 and 59.2 mol%. Therefore, the description of the genus *Lactobacillus* also needs to be amended.

Emended description of the genus *Lactobacillus* Beijerinck (1901)

The description of the genus is based on that of Beijerinck (1901), as given by Kandler & Weiss (1986), with the addition that the upper limit of the range of DNA G + C content is 59.2 mol%.

Description of *Lactobacillus nasuensis* sp. nov.

Lactobacillus nasuensis (na.su.en'sis. N.L. masc. adj. *nasuensis* named after Nasu-shiobara City, where strains were first isolated).

The species description is based on two strains. Grows well anaerobically on MRS agar at 30 °C. Cells are Gram-positive, catalase-negative, facultatively anaerobic and homofermentative, non-sporulating, non-motile short rods (8.7–9.0 µm long and 2.9–3.0 µm wide), occurring singly or in pairs. Colonies on MRS agar are white and opaque (<8.0 mm in diameter). Grows at 40 °C, at pH 4.0–8.0 and with 3.0 % NaCl, but not below 10 °C or above 45 °C,

Table 2. DNA base composition and DNA–DNA relatedness between strains SU 18^T and SU 83 and their closest phylogenetic neighbours

Values are means of three tests.

Strain	DNA G + C content (mol%)	DNA–DNA reassociation (%) with:		
		1	3	4
1. <i>Lactobacillus nasuensis</i> sp. nov. SU 18 ^T	59.2	100.0	11.3	18.8
2. <i>Lactobacillus nasuensis</i> sp. nov. SU 83	58.5	93.5	9.8	21.5
3. <i>L. manihotivorans</i> JCM 12514 ^T	48.4	18.6	100.0	15.3
4. <i>L. camelliae</i> JCM 13995 ^T	56.0	23.7	16.7	100.0

at pH 3.5 or with 6.0% NaCl. Acid is produced from ribose, but not from methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose or β -gentiobiose. With API ZYM, positive for α - and β -galactosidases, but negative for α -fucosidase. The type strain produces D- and L-isomers of lactic acid in the ratio 5.4:94.6. The DNA G + C content is 58.5–59.2 mol% (HPLC).

The type strain, SU 18^T (=JCM 17158^T =CGMCC 1.10801^T), was isolated from a Sudan grass [*Sorghum sudanense* (Piper) Stapf.] silage sample taken from the National Institute of Livestock and Grassland Science, Nasushiobara, Tochigi, Japan.

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