Lactobacillus farraginis sp. nov. and *Lactobacillus parafarraginis* sp. nov., heterofermentative lactobacilli isolated from a compost of distilled shochu residue

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Five strains of lactic acid bacteria were isolated from a compost of distilled shochu residue in Japan. The isolates were separated into two groups on the basis of 16S rRNA gene sequence similarity, and two subclusters were formed that comprised micro-organisms closely related to *Lactobacillus buchneri*, *L. diolivorans*, *L. hilgardii*, *L. kefir*, *L. parabuchneri* and *L. parakefiri*. DNA–DNA relatedness results revealed that the isolates could be separated into two groups, and these groups correlated well with the subclusters generated using the phylogenetic analysis. Moreover, the levels of DNA–DNA relatedness showed clear separation of the two groups from their phylogenetic relatives. Therefore, the two groups represent two novel species, for which the names *Lactobacillus farraginis* sp. nov. (type strain NRIC 0676^T=JCM 14108^T=DSM 18382^T) and *Lactobacillus parafarraginis* sp. nov. (type strain NRIC 0677^T=JCM 14109^T=DSM 18390^T) are proposed.

During a study of lactic acid bacteria originating from plant materials, we isolated a novel lactic acid bacterium, *Oenococcus kitaharae*, from a compost of distilled shochu residue produced in Japan (Endo & Okada, 2006). Shochu is a traditional Japanese distilled spirit made from rice, sweet potato, barley or other starchy materials; *Aspergillus niger* and *Saccharomyces cerevisiae* play roles in saccharification of the starch and its fermentation into alcohol. Concomitantly, five strains of lactic acid bacteria were isolated from the compost and were separated into two groups on the basis of their 16S rRNA gene sequences: they formed two subclusters, the members of which were related to the obligately heterofermentative lactobacilli *Lactobacillus buchneri*, *L. diolivorans*, *L. hilgardii*, *L. kefir*, *L. parabuchneri* and *L. parakefiri*. The DNA–DNA relatedness results showed clear separation of the isolates into two groups that correlated well with the two subclusters generated from a phylogenetic analysis. Moreover, the levels of DNA–DNA relatedness showed clear separation of the two groups from the phylogenetic relatives. This report provides a taxonomic analysis of the five isolates, and proposes two novel *Lactobacillus* species.

A sample of compost was prepared from a distilled shochu residue in Miyazaki Prefecture in the southern Kyushu region of Japan. The sample was serially diluted with sterile saline before being plated on MRS (Oxoid) agar containing (l⁻¹) 10 mg cycloheximide, 10 mg sodium azide, 5.0 g calcium carbonate and 15 g agar. Plates were incubated for 5 days at 30 °C. After incubation, colonies that produced clear zones were picked into MRS broth and maintained in the same medium. Of the isolates, five (NRIC 0676^T, NRIC 0677^T, NRIC 0678, NRIC 0679 and NRIC 0680) were used in the present study. Strains NRIC 0677^T and NRIC 0680 grew more slowly than the other three isolates in MRS broth; these isolates took 2–3 days to reach stationary phase, whereas NRIC 0676^T, NRIC 0678 and NRIC 0679 took 1–2 days. *L. buchneri* NRIC 1040^T, *L. diolivorans* NRIC 0695^T, *L. hilgardii* NRIC 1060^T, *L. kefir* NRIC 1693^T, *L. parabuchneri* NRIC 1780^T and *L. parakefiri* NRIC 0217^T were obtained from the NODAI Culture Collection Center, Tokyo University of Agriculture (Tokyo, Japan) and were used as reference strains in the present study. The reference strains were also maintained in MRS broth.

The 16S rRNA gene sequences of the five isolates were determined as described previously (Endo & Okada, 2005). The closest known relatives of the isolates were determined by performing DataBase searches, and the sequences of closely related species were retrieved from the DDBJ database. 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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains NRIC 0676^T, NRIC 1078, NRIC 0679, NRIC 0677^T and NRIC 0680 are AB262731–AB262735, respectively.

Maximum-likelihood and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences of the novel strains and related species are available as supplementary figures in IJSEM Online.

of levels of DNA–DNA relatedness because the 16S rRNA gene sequence similarities between the isolates and the members of these subclusters were considerably lower than the values recommended for species differentiation (Stackebrandt & Goebel, 1994). The DNA G + C contents of the novel isolates were also determined. Extraction and isolation of bacterial DNA were performed by using the method of Marmur (1961), as modified by Ezaki *et al.* (1983). DNA–DNA hybridization was carried out by using the microdilution-well technique, with photobiotin for labelling of the DNA (Ezaki *et al.*, 1989). The G + C contents of the strains tested were determined by HPLC as described by Tamaoka & Komagata (1984). The levels of DNA–DNA relatedness showed clear separation of the isolates into two groups. Strains NRIC 0676^T, NRIC 0678 and NRIC 0679 shared high levels of DNA–DNA relatedness (87–100 %), and strains NRIC 0677^T and NRIC 0680 shared 95 % DNA–DNA relatedness. In contrast, strain NRIC 0676^T showed low levels of DNA–DNA relatedness (42–46 %) with respect to NRIC 0677^T and NRIC 0680, and NRIC 0677^T also showed low levels of DNA–DNA relatedness (43–48 %) with respect to NRIC 0676^T, NRIC 0678 and NRIC 0679. Therefore, we concluded that the two groups were genetically distinguishable from each other; these groups correlated well with the two subclusters obtained using phylogenetic analysis. The levels of DNA–DNA relatedness for NRIC 0676^T with respect to *L. hilgardii* NRIC 1060^T, *L. diolivorans* NRIC 0695^T, *L. buchneri* NRIC 1040^T, *L. parabuchneri* NRIC 1780^T, *L. kefir* NRIC 1693^T and *L. parakefir* NRIC 0217^T were 47, 35, 30, 29, 23 and 20 %, respectively; those for NRIC 0677^T with respect to these reference strains were 37, 32, 25, 25, 30 and 21 %, respectively. The G + C contents of strains NRIC 0676^T, NRIC 0678, NRIC 0679 ranged from 40 to 41 mol% and that of strains NRIC 0677^T and NRIC 0680 was 40 mol%.

Randomly amplified polymorphic DNA (RAPD) fingerprinting was performed to differentiate the five isolates by a method described previously (Endo & Okada, 2006). Primers D (5'-GAGGACAAAG), E (5'-GGCGTCGGTT) and F (5'-GGCCACGGAA) were used in this study. The fingerprinting indicated that the isolates were separated into two groups. The fingerprints of strains NRIC 0676^T, NRIC 0678 and NRIC 0679 differed slightly, and those of strains NRIC 0677^T and NRIC 0680 were similar to each other (Fig. 2). This demonstrated that the isolates were separated at the strain level within each group. The two groups produced fingerprints that were quite different. The groups correlated well with the separate groups generated by phylogenetic analysis or levels of DNA–DNA relatedness.

Morphological, physiological and biochemical characteristics of the isolates were determined by using a method described previously (Endo & Okada, 2005); MRS broth was used as a basal medium. The characteristics of the isolates (described in detail in the species descriptions below) were compared with those of closely related species (Table 1). Members of the *L. brevis* and *L. fructivorans* subclusters

Fig. 2. RAPD PCR fingerprinting of the five novel isolates. Primers D, E and F were used. Lanes: M, size markers (500 bp DNA ladder; Takara-bio); 1, NRIC 0676^T; 2, NRIC 0678; 3, NRIC 0679; 4, NRIC 0677^T; 5, NRIC 0680.

were not used for comparisons with the isolates because of the low levels of 16S rRNA gene sequence similarity to the novel strains. The isolates were separated into two groups on the basis of phenotypic characteristics, and these groups correlated well with the two groups separated on the basis of phylogenetic analysis, levels of DNA–DNA relatedness and RAPD fingerprinting. Strains NRIC 0676^T, NRIC 0677^T and NRIC 0678 grew at 45 °C and grew slowly at 15 °C, but strains NRIC 0677^T and NRIC 0680 did not grow at 15 or 45 °C. Growth at 45 °C was not found in other species of

Table 1. Differential characteristics among the novel isolates and closely related species

Taxa: 1, strains NRIC 0676^T, NRIC 0678 and NRIC 0679 (*L. farraginis* sp. nov.); 2, strains NRIC 0677^T and NRIC 0680 (*L. parafarraginis* sp. nov.); 3, *L. buchneri* (data from Kandler & Weiss, 1986); 4, *L. diolivorans* (Krooneman *et al.*, 2002); 5, *L. hilgardii* (Kandler & Weiss, 1986); 6, *L. kefir* (Kandler & Kunath, 1983); 7, *L. parabuchneri* (Farrow *et al.*, 1988); 8, *L. parakefir* (Takizawa *et al.*, 1994). +, Positive; –, negative; V, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Acid from:								
L-Arabinose	+	+	+	+	–	+	+	+
D-Xylose	–	+	V	+	+	–	–	–
Galactose	+	+	V	+	V	–	+	+
Lactose	–	–	V	–	V	+	–	+
Melibiose	+	+	+	+	–	+	+	V
Sucrose	+	+	V	–	V	–	+	–
Raffinose	+	+	V	V	–	–	–	–
Melezitose	+	+	+	–	V	–	+	V
Growth at:								
10 °C	–	–	ND	–	ND	+	+	ND
45 °C	+	–	–	–	–	–	–	–

the *L. buchneri* subcluster (Table 1). However, as the major characteristics of the strains in the two groups were similar to those of the phylogenetic relatives, precise identification among the two groups and their phylogenetic relatives based on phenotypic features is difficult. Therefore, determinations of DNA–DNA relatedness are needed for precise identification of the species in the *L. buchneri* subcluster. The same has been observed for the species in the *Lactobacillus acidophilus* group (Fujisawa *et al.*, 1992; Naser *et al.*, 2006).

The data showed that the five isolates could be divided into two genetically distinct groups; these groups were also genetically distinguishable from known species of lactic acid bacteria. Thus, these two groups represent two novel species, for which the names *Lactobacillus farraginis* sp. nov. (strains NRIC 0676^T, NRIC 0678 and NRIC 0679) and *Lactobacillus parafarraginis* sp. nov. (strains NRIC 0677^T and NRIC 0680) are proposed.

Description of *Lactobacillus farraginis* sp. nov.

Lactobacillus farraginis (far.ra'gi.nis. L. gen. n. *farraginis* of mash, pertaining to shochu mash, an ingredient of a compost material from which the type strain was isolated).

Cells are Gram-positive, non-motile rods, measuring $0.8 \times 3\text{--}6\ \mu\text{m}$. Cells occur singly or in pairs and chains. Facultatively anaerobic and catalase-negative. Colonies on MRS agar are beige, smooth and approximately 2 mm in diameter after incubation for 4 days. Heterofermentative and produces lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. D- and L-lactate are produced in a ratio of 1 : 1. Nitrate is not reduced. Acid is produced from D-glucose, D-fructose, D-galactose, L-arabinose, D-ribose, maltose, melibiose, sucrose, raffinose and D-melezitose, weakly from D-gluconate, but not from D-mannose, D-xylose, L-rhamnose, cellobiose, lactose, D-salicin, D-trehalose, D-mannitol, D-sorbitol or starch. Dextran is not formed from sucrose. Cells grow at temperatures between 15 and 45 °C but not at 10 or 50 °C. All strains grow at pH 4.0–8.5; some strains grow at pH 9.0. No growth is observed in MRS broth containing 5 % (w/v) NaCl. Cells do not contain *meso*-diaminopimelic acid in their peptidoglycan. The DNA G + C content ranges from 40 to 41 mol%.

The type strain is NRIC 0676^T (=JCM 14108^T=DSM 18382^T), and has a G + C content of 41 mol%. All three known strains were isolated from a compost of distilled shochu residue collected at a shochu distillery in Miyazaki Prefecture, southern Kyushu region, Japan, in 2003.

Description of *Lactobacillus parafarraginis* sp. nov.

Lactobacillus parafarraginis (pa.ra.far.ra'gi.nis. Gr. prep. *para* beside; L. gen. n. *farraginis* of mash, the specific epithet of *Lactobacillus farraginis*; N.L. gen. n. *parafarraginis* beside *farraginis*, pertaining to the close relationship to *L. farraginis*).

Cells are Gram-positive, non-motile rods, measuring $0.8 \times 2\text{--}4\ \mu\text{m}$. Cells occur singly or in pairs and chains. Facultatively anaerobic and catalase-negative. Colonies on MRS agar are beige, smooth and approximately 1–2 mm in diameter after incubation for 4 days. Heterofermentative and produces lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. D- and L-lactate are produced in the ratio 3 : 2. Nitrate is not reduced. Acid is produced from D-glucose, D-fructose, D-galactose, L-arabinose, D-ribose, D-xylose, maltose, melibiose, sucrose, raffinose and D-melezitose, weakly from D-gluconate, but not from D-mannose, L-rhamnose, cellobiose, lactose, D-salicin, D-trehalose, D-sorbitol or starch. Acid production from D-mannitol is variable depending on the strain. Dextran is not formed from sucrose. Cells grow at temperatures between 20 and 37 °C but not at 15 or 45 °C. All strains grow at pH 4.0–8.5. Growth is observed in MRS broth containing 5 % (w/v) NaCl. Cells do not contain *meso*-diaminopimelic acid in their peptidoglycan. The DNA G + C content is 40 mol%.

The type strain is NRIC 0677^T (=JCM 14109^T=DSM 18390^T), and has a G + C content of 40 mol%. Both known strains were isolated from a compost of distilled shochu residue collected at a shochu distillery in Miyazaki Prefecture, southern Kyushu area, Japan, in 2003.

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