Lactobacillus concavus sp. nov., isolated from the walls of a distilled spirit fermenting cellar in China

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Two novel *Lactobacillus* strains, C-5-1^T and HB5, were isolated from the walls of a distilled spirit fermenting cellar in Hebei province, China. The strains were Gram-positive, non-spore-forming, non-motile curved rods and were facultatively anaerobic. They produced 5% (–)-D/95% (+)-L lactic acid exclusively from glucose fermentation, but were catalase-negative. Both strains fermented *N*-acetylglucosamine, arbutin, salicin, cellobiose, maltose, trehalose and β -gentiobiose. The mean DNA G+C content of the two strains was 46.9 ± 0.8 mol% (46.4 mol% for the type strain, C-5-1^T). Phylogenetic analysis based on 16S rRNA gene sequence similarity showed that strain C-5-1^T was clustered in the *Lactobacillus casei*/*Pediococcus* phylogenetic group and was closely related to *Pediococcus dextrinicus* JCM 5887^T (97.9% similarity), followed by *Lactobacillus algidus* JCM 10491^T (93.9% similarity). The DNA–DNA relatedness between the type strain C-5-1^T and *P. dextrinicus* JCM 5887^T and *L. algidus* JCM 10491^T was only 5.4 and 4.3%, respectively. Therefore, based on phenotypic, genotypic and phylogenetic analyses, a novel *Lactobacillus* species, *Lactobacillus concavus* sp. nov., is proposed. The type strain is C-5-1^T (= AS 1.5017^T = LMG 22739^T).

Since the genus *Lactobacillus* was originally described by Beijerinck (Beijerinck, 1901; Skerman *et al.*, 1980), nearly 120 species have been recognized. The lactobacilli are widely distributed in the environment and are frequently found in fermented food, vegetables and in the intestines of man and animals (Omar *et al.*, 2000; Gardner *et al.*, 2001; Satokari *et al.*, 2003). Lactobacilli also play important roles in the wine-making process; they are responsible for malolactic fermentation, which follows alcoholic fermentation by yeasts (Lonvaud-Funel, 1999). In recent years, some novel lactic acid bacteria, such as *Lactobacillus ferintoshensis* (Simpson *et al.*, 2001), *Lactobacillus nagelii* (Edwards *et al.*, 2000), *Lactobacillus amylolyticus* (Bohak *et al.*, 1998) and *Pediococcus claussenii* (Dobson *et al.*, 2002), have been isolated from malt whisky, grape wine, beer malt and beer, respectively.

Chinese liquors are a type of wine produced through a solid fermentation of grain. During this process, grains of wheat or other cereals are mixed with water and sealed in a fermenting cellar for several months. The grains are fermented by the microorganisms present in the mud wall of the cellar. During an investigation of lactic acid bacteria in Chinese

wine breweries, we isolated two *Lactobacillus* strains from a brewery in Hebei province, China, which exhibited phenotypic characteristics that distinguished them from all the *Lactobacillus* species with validly published names. Phylogenetic analysis based on 16S rRNA gene sequence similarity and DNA–DNA hybridization indicated that the strains could represent a novel species of the genus *Lactobacillus*.

Lactobacillus algidus JCM 10491^T (Kato *et al.*, 2000) was provided by JCM (Japan Collection of Microorganisms). Strains C-5-1^T and HB5 were originally isolated from wall samples from a distilled spirit fermenting cellar in Hebei province, China. The samples were cultured anaerobically in MRS liquid (de Man *et al.*, 1960) at 37 °C for 2 days and then purified on MRS agar. The strains were routinely grown anaerobically on the same medium at 37 °C unless otherwise stated. The end products of glucose fermentation in tryptone peptone yeast-extract glucose (TPYG) medium (Scardovi, 1986) were detected using a gas chromatograph (GC-14B; Shimadzu). Isomers of lactate formed from glucose were determined using a D-L-lactic acid test kit (Roche Diagnostics). Catalase activity was determined by adding 15% (v/v) hydrogen peroxide to a fresh culture on a glass slide. The temperature profile was determined by using a water bath with a temperature gradient. Growth at various pH values was investigated using TPYG liquid medium adjusted with hydrochloric acid or NaOH solution. Tolerance to ethanol was measured by monitoring growth in TPYG liquid medium containing various concentrations (v/v) of

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Abbreviation: DAP, 2, 6-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lactobacillus concavus* sp. nov. AS 1.5017^T is AY683322.

Table 1. Differential phenotypic characteristics of *Lactobacillus concavus* sp. nov. and phylogenetically related species

Species: 1, *L. concavus* C-5-1^T; 2, *L. concavus* HB5; 3, *L. algidus* (data from Kato *et al.*, 2000); 4, *L. durianis* (Leisner *et al.*, 2002); 5, *L. ferintoshensis* (Simpson *et al.*, 2001); 6, *P. dextrinicus* (Back, 1978). DNA G+C content was determined by the temperature melting method unless otherwise indicated. The percentage of the (+)-L isomer of lactic acid present is indicated by DL, 40–60 % (+)-L-lactic acid; L+DL, 60–80 % (+)-L-lactic acid; L, 80–100 % (+)-L-lactic acid. +, More than 90 % of strains positive; –, <10 % of strains positive; D+, 50–89 % of strains positive; D–, 11–49 % of strains positive; W, weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6
Cell morphology	Rod	Rod	Rod	Rod	Rod	Round
Gas from gluconate	–	–	–	+	–	+
Growth at:						
30 °C	+	+	–	+	+	+
45 °C	–	–	–	–	–	–
Acid production from:						
Amygdalin	–	+	D–	–	ND	+
L-Arabinose	–	–	+	+	+	+
D-Fructose	–	+	+	–	+	+
Galactose	–	–	+	D+	+	+
Gluconate	–	–	–	W	–	+
D-Mannose	–	–	+	–	–	–
Ribose	–	–	+	+	+	–
NH ₃ from arginine	–	–	–	–	+	–
Lactic acid isomer	L	L	L	L+DL	DL	L
DNA G+C content (mol%)	46.4	47.5	36.5* 37.2†	43.2	ND	40.6

*Determined by HPLC.

†This study.

ethanol. Generation time was measured by monitoring the OD₆₀₀ of the MRS culture at 37 °C for 72 h. The sugar fermentation pattern was determined using the API 50 CH system (bioMérieux) and 24 h incubation at 37 °C. The cell-wall peptidoglycan was of the diaminopimelic acid (DAP) type as determined using a method modified from Becker *et al.* (1964). All of the tests were performed in duplicate.

Genomic DNA was extracted and purified using a modified method of Marmur (1961) and Dong *et al.* (2000). The 16S rRNA gene was amplified by PCR using genomic DNA as template. PCR products were sequenced by using a Dye terminator cycle sequencing ready reaction kit with an ABI PRISM 377XL DNA sequencer. The closest matching sequences were retrieved from the GenBank database and aligned. Similarity analysis was performed using the CLUSTAL_X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) implemented in MEGA2 (Kumar *et al.*, 2001). The stability of the tree was evaluated by bootstrap analysis of 1000 datasets. DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). DNA–DNA relatedness was determined on the basis of the DNA–DNA liquid hybridization rate (De Ley *et al.*, 1970) at 63 °C using a spectrophotometer with a thermal controller (DU-800; Beckman).

The two isolates were Gram-positive, non-spore-forming, non-motile curved rods. They had no catalase activity, were facultatively anaerobic and produced lactic acid exclusively [about 5 % (–)-D and 95 % (+)-L] without gas from glucose fermentation. The strains grew on acetate agar (pH 5.4) and did not produce H₂S. These characters demonstrated that they were members of the genus *Lactobacillus*. By using the API 50 CH system, the two novel strains were found to ferment only a few types of sugars (Table 1 and species description). Strains C-5-1^T and HB5 showed different reactions for the fermentation of D-fructose and amygdalin. The mean generation time of the two strains was 2.3 ± 0.1 h when cultured anaerobically and 3.6 ± 0.3 h when cultured aerobically. As expected from their habitat, the two strains were able to grow in concentrations of 8 % (v/v) ethanol.

The complete 16S rRNA gene sequence of strain C-5-1^T was compared with the eight most similar sequences retrieved from GenBank. Six of these sequences were from members of genus *Lactobacillus* and the remaining two were from members of the genus *Pediococcus*. A phylogenetic tree (Fig. 1) rooted with *Bacillus subtilis* NCDO 1769^T was constructed using the neighbour-joining method. Strain C-5-1^T was found to be clustered in the *L. casei*/*Pediococcus*

Fig. 1. Dendrogram based on 16S rRNA gene sequence similarity of a consensus length of 1336 bp, indicating the phylogenetic relationship of *Lactobacillus concavus* C-5-1^T and nine other bacterial species. The tree was rooted with *Bacillus subtilis* NCDO 1769^T and constructed by using the neighbour-joining method with bootstrap values calculated from 1000 trees. The numbers at each branch point are the percentages supported by bootstrap. GenBank numbers are in parentheses. Bar, 1 % sequence divergence.

phylogenetic group (Collins *et al.*, 1991) with 16S rRNA gene sequence similarity ranging from 87.3 to 97.9 %. Strain C-5-1^T had the highest 16S rRNA gene sequence similarity to *Pediococcus dextrinicus* JCM 5887^T (97.9 %). The highest similarity to a *Lactobacillus* species, *L. algidus* JCM 10491^T, was only 93.9 %. The stability of the clustering in the tree (Fig. 1) was supported by high bootstrap values (≥ 80 %). Therefore, strain C-5-1^T may represent a novel *Lactobacillus* species belonging to the *L. casei*/*Pediococcus* phylogenetic group.

The mean DNA G + C content of the two novel strains was 46.9 ± 0.8 mol% (46.4 mol% for the type strain C-5-1^T), which was within the range characteristic for the genus *Lactobacillus* (32–55 mol%). However, this value was about 10 mol% higher than that of its phylogenetically closest *Lactobacillus* species, *L. algidus* JCM 10491^T (36 mol%). It is generally agreed that organisms exhibiting a difference in DNA G + C content of more than 2 mol% might be members of different species (Johnson, 1973). The DNA–DNA hybridization rate between strains C-5-1^T and HB5 was determined to be 100 %, indicating that they formed a homogeneous genetic group. However, the DNA–DNA relatedness between strain C-5-1^T and the phylogenetically closest species, *L. algidus* JCM 10491^T and *P. dextrinicus* JCM 5887^T, was 4.3 and 5.4 %, respectively; much lower than the DNA–DNA relatedness threshold of 70 % suggested to delineate a species.

Although strain C-5-1^T shared the highest 16S rRNA gene sequence similarity to *P. dextrinicus* JCM 5887^T (97.9 %), the following characteristics verified that the two novel strains constituted a separate species. Strain C-5-1^T was a curved rod, while *P. dextrinicus* was spherical and occurred in tetrads. *P. dextrinicus* fermented D-fructose, amygdalin, galactose, D-mannose and gluconate (acid and gas) but strain C-5-1^T did not (Table 1). Strain C-5-1^T and *P. dextrinicus* showed low values of DNA–DNA relatedness and had a DNA G + C content which differed by about 6 mol%. The two novel strains could be easily differentiated from the closely related *Lactobacillus* species, *L. algidus*, a psychrophilic bacterium isolated from refrigerated beef, as they were able to grow above 30 °C, were unable to ferment ribose, galactose, L-arabinose or D-mannose and had a 10 mol% difference in DNA G + C content (Table 1).

Collins *et al.* (1991) showed that *P. dextrinicus* formed a peripheral branch of the genus *Pediococcus* with low 16S rRNA gene sequence similarity (92.6–93 %), but that it was intermixed in the 16S rRNA phylogenetic tree with species of *Lactobacillus* with even higher 16S rRNA gene sequence similarity (92.0–94.8 %). This implied that *P. dextrinicus* could be a specific lactic acid coccus and that its taxonomic position might need to be reconsidered taking into account its ability to ferment different substrates, such as dextrin and starch, when compared with other *Pediococcus* species. Recently, it has been found that bacterial cell shape is controlled by only a few genes, such as *mreB* and *fstZ*, and

an alteration in cell shape can be induced by the inactivation of a single gene in this group (Jones *et al.*, 2001). Hence, it may not be appropriate to use cell shape as a taxonomic marker in some groups of bacteria such as *Lactobacillus* and *Pediococcus*.

On the basis of a combination of phenotypic characteristics, phylogenetic relationships and DNA–DNA relatedness, a novel species of the genus *Lactobacillus*, *Lactobacillus concavus* sp. nov., is proposed.

Description of *Lactobacillus concavus* sp. nov.

Lactobacillus concavus (con.ca'vus. L. adj. *concavus* curved, referring to the curved shape of the strains).

Cells are Gram-positive, non-spore-forming, non-motile curved rods, about $0.5\text{--}0.6 \times 2.0\text{--}2.5$ μm in size after 24 h incubation in anaerobic MRS liquid medium. Colonies are white, convex and smooth with an entire margin. Colonies are about 1 mm in diameter after 24 h cultivation on anaerobic MRS plates. Catalase-negative. Facultatively anaerobic. Lactic acid [about 5 % (–)-D and 95 % (+)-L], but no gas, is produced from glucose and gluconate fermentation. The diagnostic diamino acid in the cell-wall peptidoglycan is *meso*-DAP. The optimum temperature for growth is 30–37 °C; the temperature ranges for growth for C-5-1^T and HB5 are 10–42 °C and 10–39 °C, respectively. Optimum pH is 6.0–6.4; the pH ranges for growth of C-5-1^T and HB5 are 3.8–8.1 and 4.3–8.1, respectively. Growth can occur in 8 % (v/v) ethanol, but not in 6.5 % (w/v) NaCl. Aesculin is hydrolysed but arginine is not hydrolysed. Voges–Proskauer test is negative. Acid is produced from D-glucose, N-acetylglucosamine, arbutin, salicin, cellobiose, maltose, trehalose and β -gentiobiose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -xyloside, galactose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Fermentation of D-fructose and amygdalin is variable. The DNA G + C content is 46.9 ± 0.8 mol% (46.4 mol% for the type strain, C-5-1^T).

The type strain, C-5-1^T (= AS 1.5017^T = LMG 22739^T), was isolated from the walls of a distilled spirit fermenting cellar in Hebei province, China.

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