Lactobacillus similis sp. nov., isolated from fermented cane molasses

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The taxonomic position of strain JCM 2765^T isolated from fermented cane molasses in Thailand was reinvestigated. Strain JCM 2765^T was originally identified as representing *Lactobacillus buchneri* on the basis of biochemical and physiological characteristics. In the present study, 16S rRNA gene sequence analysis of strain JCM 2765^T demonstrated a low level of similarity with the type strain of *L. buchneri* (92.5%) and high levels with those of *Lactobacillus collinoides* (97.6%) and *Lactobacillus paracollinoides* (98.0%). Ribotyping was applied to investigate the relationships between strain JCM 2765^T, *L. collinoides* and *L. paracollinoides*. The dendrogram based on ribotyping patterns showed one cluster for six strains of *L. paracollinoides*, and that strain JCM 2765^T and *L. collinoides* JCM 1123^T were each independent. Based on additional phenotypic findings and DNA–DNA hybridization results, strain JCM 2765^T is considered to represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus similis* sp. nov. is proposed. The type strain is JCM 2765^T (=LMG 23904^T).

Strain JCM 2765^T was one of 89 isolates from fermented cane molasses at alcohol plants in Thailand and was first described by Seki *et al.* (1989). Strain JCM 2765^T was a facultatively anaerobic, non-spore-forming, Gram-positive rod-shaped bacterium, and produced lactic acid from glucose as a major product. It was identified by Seki *et al.* (1989) as representing *Lactobacillus buchneri* on the basis of biochemical and physiological characteristics compared with *L. buchneri* JCM 1115^T. In the present study, further genomic research revealed that strain JCM 2765^T represents a novel species of the genus *Lactobacillus*.

The following eight strains were investigated: JCM 2765^T, *Lactobacillus collinoides* JCM 1123^T, and *Lactobacillus paracollinoides* JCM 11969^T (=LA2^T), JCM 1113, JCM 15728 (=LA3), JCM 15729 (=LA4), JCM 15730 (=LA7) and JCM 15731 (=LA8). The above *L. paracollinoides* strains were isolated from brewery environment and were described by Suzuki *et al.* (2004a, b, c, 2006). Strain JCM 2765^T and *L. collinoides* JCM 1123^T were grown on MRS (Merck) agar plates at 37 and 26 °C, respectively, under

anaerobic conditions. *L. paracollinoides* strains were grown in beer medium agar plates at 26 °C under anaerobic conditions. Beer medium was prepared as MRS medium dissolved with normally hopped beer instead of distilled water.

Physiological, biochemical and enzyme activity tests were performed by inoculation of API 50 CH and API ZYM (bioMérieux) strips in triplicate according to the manufacturer's instructions. Production of CO₂ from glucose fermentation, catalase activity, determination of D- and L-lactic acid production, and aesculin hydrolysis were reported by Seki *et al.* (1989). Approximately 1500 bases of the 16S rRNA gene sequences of strains JCM 2765^T, JCM 11969^T, JCM 1113 and JCM 15728–15731 were amplified by PCR with universal primers 27F (5'-dAGAGTTT-GATCCTGGCTCAG-3') and 1492R (5'-dGGTTACCTT-GTTACGACTT-3') by using a Biometra Thermocycler Tgradient. PCR products were purified by using an Ultraclean PCR Clean-up kit (Mo Bio) and were sequenced by using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The closest recognized relatives of the isolates were determined by performing database searches, and sequences of closely related species were retrieved from the DDBJ, EMBL and GenBank nucleotide sequence databases. Phylogenetic analysis was performed with the CLUSTAL X (version 1.83) program (Thompson *et al.*, 1997) and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by bootstrap analysis of 1000 replicates by using

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JCM 2765^T, JCM 11969^T, JCM 1113, JCM 15728, JCM 15729, JCM 15730 and JCM 15731 are AB282889 and AB473623–AB473628, respectively.

Minimum-evolution and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences showing the relationship between strain JCM 2765^T and related species, table giving differential characteristics between strain JCM 2765^T, *Lactobacillus collinoides* and *Lactobacillus paracollinoides*, and table giving levels of DNA–DNA relatedness between strain JCM 2765^T and strains of *L. collinoides* and *L. paracollinoides* are available with the online version of this paper.

the CLUSTAL X software. Minimum-evolution and maximum-parsimony phylogenetic trees were inferred by using the software package MEGA, version 4.0 (Tamura *et al.*, 2007), according to the Kimura two-parameter model (Kimura, 1980). A species-specific PCR assay to identify *L. paracollinoides* was performed according to Suzuki *et al.* (2004b). The automated ribotyping device RiboPrinter microbial characterization system (Qualicon) was used for ribotyping in accordance with the manufacturer's instructions, with *EcoRI* as a restriction enzyme. Ribopatterns were analysed by BioNumerics version 2.5 software (Applied Maths) and were compared by Pearson similarity coefficient analysis and the unweighted pair group method using arithmetic average (UPGMA) algorithm. For DNA–DNA hybridization experiments, bacterial DNA of seven strains, JCM 2765^T, *L. collinoides* JCM 1123^T, and *L. paracollinoides* JCM 11969^T, JCM 1113, JCM 15728, JCM 15729 and JCM 15730, was extracted from cells harvested from MRS broth (Difco) after 12 h as described by Kitahara *et al.* (2001) and was purified according to the methods of Saito & Miura (1963). Levels of DNA–DNA relatedness were determined according to the method of Ezaki *et al.* (1989) by using photobiotin and microplates. Hybridization was performed at 45 °C for 18 h. The DNA G + C content was determined as described by Kitahara *et al.* (2001).

Approximately 1500 bases of the 16S rRNA gene sequence of strain JCM 2765^T were determined. The neighbour-joining, minimum-evolution and maximum-parsimony phylogenetic trees clearly indicated that strain JCM 2765^T was related to strains in the genus *Lactobacillus*, as shown in Fig. 1 and Supplementary Figs S1 and S2 available in IJSEM Online. The phylogenetic trees showed clearly that strain JCM 2765^T had a distinct line of descent with high bootstrap support. Strain JCM 2765^T showed highest 16S rRNA gene sequence similarity to *L. collinoides* JCM 1123^T (97.6 %) and *L. paracollinoides* JCM 11969^T (98.0 %). Strain JCM 2765^T was originally identified by Seki *et al.* (1989) as representing *L. buchneri* based on biochemical and physiological characteristics, but the two taxa were not closely related in the present phylogenetic trees; 16S rRNA gene sequence similarity between strain JCM 2765^T and the type strain of *L. buchneri* was 92.5 %. In the present study, 16S rRNA gene sequences for six strains of *L. paracollinoides* were redetermined, and their sequence similarities were over 99.9 %. Strains JCM 11969^T, JCM 15728, JCM 15729 and JCM 15731 had identical sequences. The level of 16S rRNA gene sequence similarity between *L. paracollinoides* JCM 11969^T and *L. collinoides* JCM 1123^T was 99.6 % in the present study. However, *L. paracollinoides* can be differentiated from *L. collinoides* on the basis of

Fig. 1. Phylogenetic tree showing the relationship between strain JCM 2765^T and representatives of related species. The tree was constructed by the neighbour-joining method based on 16S rRNA gene sequences. Numbers at nodes indicate percentage bootstrap values of 1000 replicates; only values >50 % are given. Accession numbers for 16S rRNA gene sequences are given in parentheses for each strain. Bar, 0.01 substitutions per nucleotide position. Minimum-evolution and maximum-parsimony trees are available as Supplementary Figs S1 and S2 in IJSEM Online.

DNA–DNA relatedness and beer-spoiling ability (Suzuki *et al.*, 2004a). Furthermore, species-specific primers based on intergenic spacer region sequences were published for use in the differentiation of beer-spoiling *L. paracollinoides* from non-beer-spoiling *L. collinoides* strains (Ehrmann & Vogel, 2005; Suzuki *et al.*, 2004b). In the present study, strain JCM 2765^T had the same amplified band pattern as *L. paracollinoides* strains based on the specific primers for *L. paracollinoides* (data not shown), although *L. collinoides* JCM 1123^T had a different amplified band pattern as reported by Ehrmann & Vogel (2005). The specific primer for *L. paracollinoides* was not effective for differentiation between strain JCM 2765^T and *L. paracollinoides*.

Ribotyping via the RiboPrinter system was applied to investigate the relationships between strain JCM 2765^T, *L. collinoides* and *L. paracollinoides* (Fig. 2). The dendrogram by ribotyping patterns showed one cluster for six strains of *L. paracollinoides*, and that strain JCM 2765^T and *L. collinoides* JCM 1123^T were each independent. Moreover, the ribotyping also showed that the six strains of *L. paracollinoides* fell into two distinct clusters as in the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1). Four strains of *L. paracollinoides*, JCM 11969^T, JCM 15728, JCM 15729 and JCM 15731, had similar ribotyping patterns, consistent with levels of 16S rRNA gene sequence similarity.

Physiological and biochemical properties of strain JCM 2765^T were determined via the API 50 CH and API ZYM systems. The results are given in the species description below. Identical results were shown for strain JCM 2765^T and *L. collinoides* JCM 1123^T with the API ZYM system, but contrasting results were shown by the API 50 CH system among strain JCM 2765^T, *L. collinoides* JCM 1123^T and *L. paracollinoides* strains. The combination of carbohydrate utilization can be used to differentiate among the three taxa. For example, strain JCM 2765^T showed positive and negative reactions for utilization of galactose and D-fructose, respectively, whereas those for *L. collinoides* JCM 1123^T were both positive, and those for *L. paracollinoides* were both negative (Supplementary Table S1, available in IJSEM Online).

DNA–DNA hybridization experiments were performed among strain JCM 2765^T, *L. collinoides* JCM 1123^T and *L. paracollinoides* JCM 11969^T, JCM 1113, JCM 15728, JCM 15729 and JCM 25730 (Supplementary Table S2, available in IJSEM Online). Low levels of DNA–DNA hybridization were found between strain JCM 2765^T and *L. collinoides* JCM 1123^T (6%), and also between strain JCM 2765^T and *L. paracollinoides* strains (7–16%). These low values indicated clearly that strain JCM 2765^T belongs to a species distinct from *L. collinoides* and *L. paracollinoides*. Suzuki *et al.* (2004a) reported that levels of DNA–DNA relatedness values between *L. collinoides* JCM 1123^T and *L. paracollinoides* strains were relatively low (46.8–57.6%), but values in the present study were much lower (22–31%). These differences might be related to the hybridization temperature, but no information about hybridization temperature was reported by Suzuki *et al.* (2004a). Agreement among the two hybridization results in the present study (Supplementary Table S2), fixed DNA side and photobiotin-labelled side, supported the reliability of our data. The DNA G + C content of strain JCM 2765^T was 48.5 mol %, which is within the range reported for the genus *Lactobacillus* (32–53 mol%) (Kandler & Weiss, 1986). On the basis of the results presented here, strain JCM 2765^T is considered to represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus similis* sp. nov. is proposed.

Description of *Lactobacillus similis* sp. nov.

Lactobacillus similis (si'mi.lis. L. masc. adj. *similis* similar, resembling, as the type strain is similar to those of *L. collinoides* and *L. paracollinoides*).

Cells cultivated on MRS agar plates are Gram-positive, non-spore-forming, non-motile rods. Cells are 0.5 µm in width and variable in length, mostly ranging between 2 and 5 µm. Colonies are 1–2 mm in diameter, disc-shaped and white when grown on MRS agar plates at 37 °C for 2 days. The optimal growth temperature is 37 °C. Grows at 15 °C but not at 45 °C. Facultatively anaerobic, produces gas from glucose, and produces D- and L-lactic acids heterofermentatively. Catalase is not produced. Aesculin is not

Fig. 2. Dendrogram illustrating the relatedness of the ribotyping patterns of strain JCM 2765^T, and *L. collinoides* and *L. paracollinoides* strains. The dendrogram was analysed via Pearson similarity coefficient analysis and the UPGMA algorithm.

hydrolysed. In the API 50CH system, acid is produced from L-arabinose, ribose, D-xylose, methyl β -D-xyloside, galactose, D-glucose, N-acetylglucosamine, maltose, lactose, melibiose and gluconate. Variable reactions with raffinose and 5-ketogluconate. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, sucrose, trehalose, inulin, melezitose, starch, glycogen, xylitol, β -gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol or 2-ketogluconate. In the API ZYM system, positive for esterase, esterase lipase, leucine arylamidase, valine arylamidase, phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase and β -glucosidase. Negative reactions for alkaline phosphatase, lipase, cystine arylamidase, trypsin, chymotrypsin, β -glucuronidase, N-acetyl- β -glucosamidase, α -mannosidase and α -fucosidase.

The DNA G + C content of strain JCM 2765^T is 48.5 %. The type strain is JCM 2765^T (=LMG 23904^T), isolated from fermented cane molasses at alcohol plants in Thailand.

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