Leuconostoc durionis sp. nov., a heterofermenter with no detectable gas production from glucose

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Three lactic acid bacterial (LAB) strains obtained from a Malaysian acid-fermented condiment, tempoyak (made from pulp of the durian fruit), showed analogous but distinct patterns after screening by SDS-PAGE of whole-cell proteins and comparison with profiles of all recognized LAB species. 16S rRNA gene sequencing of one representative strain showed that the taxon belongs phylogenetically to the genus *Leuconostoc*, with its nearest neighbour being *Leuconostoc fructosum* (98 % sequence similarity). Biochemical characteristics and DNA–DNA hybridization experiments demonstrated that the strains differ from *Leuconostoc fructosum* and represent a single, novel *Leuconostoc* species for which the name *Leuconostoc durionis* sp. nov. is proposed. The type strain is LMG 22556^T (=LAB 1679^T=D-24^T=CCUG 49949^T).

During a study of the biodiversity of the microflora of tempoyak, a traditional Malaysian acid-fermented condiment derived from pulp of the durian fruit, a collection of lactic acid bacterial (LAB) isolates was recovered on MRS (de Man–Rogosa–Sharpe; Oxoid) agar with 0.14 % sorbic acid (MRS-S). The strains were identified by using SDS-PAGE of whole-cell proteins as described by Pot *et al.* (1994). For identification, strains were cultivated on MRS agar at 30 °C for 24 h. Whole-cell protein extracts were prepared and SDS-PAGE was performed. A densitometric analysis, normalization and interpolation of the protein profiles, and a numerical analysis were performed by using the GELCOMPAR software package versions 3.1 and 4.0, respectively (Applied Maths). The whole-cell protein profiles of three tempoyak isolates, designated LMG 22556^T (=LAB 1679^T=D-24^T), LMG 22557 (=LAB 1663=D-6) and LMG 22558 (=LAB 1674=D-18), indicated that they represented a homogeneous taxon but occupied a separate position (see supplementary figure in IJSEM Online) and

remained unidentified after comparison with patterns representing all recognized LAB species (Pot & Janssens, 1993; data not shown).

The phylogenetic position of one representative strain, LMG 22556^T, was determined by 16S rRNA gene sequence analysis. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). The 16S rRNA gene sequence was amplified using oligonucleotide primers complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer was 5'-AGAGTTT-GATCCTGGCTCAG-3' (hybridizing at positions 8–27, according to the *Escherichia coli* numbering system) and the reverse primer was 5'-AAGGAGGTGATCCAGCCGCA-3' (hybridizing at positions 1541–1522). PCR products were purified using NucleoFast 96 PCR plates (Macherey-Nagel) according to the manufacturer's instructions. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and purified using the Montage SEQ₉₆ Sequencing Reaction Cleanup kit (Millipore), based on the protocols recommended by the manufacturers. Sequencing was achieved using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The eight sequencing primers used are listed in Coenye *et al.* (1999). Sequence assembly was performed using the program AUTOASSEMBLER (Applied Biosystems). The 16S rRNA gene sequence (a continuous stretch of 1502 bp) and

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of LMG 22556^T is AJ780981.

Protein profiles and a corresponding dendrogram of *Leuconostoc durionis* and other *Leuconostoc* species are available as a supplementary figure in IJSEM Online.

sequences of strains retrieved from EMBL were aligned, and a phylogenetic tree was constructed by the neighbour-joining method using the BIONUMERICS 3.50 software package (Applied Maths). Unknown bases were discarded for the analyses. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data (Fig. 1). Comparison with deposited sequences available in the EMBL database classified the strain within the genus *Leuconostoc*; its nearest neighbour was *Leuconostoc fructosum* (sequence similarity of 98.0%). 16S rRNA gene sequence similarities to other *Leuconostoc* species were in the range 91.0–94.4%.

DNA base compositions were determined for the three new isolates and for *Leuconostoc fructosum* LMG 9498^T. Strains were cultivated on MRS agar at 30 °C for 24 h. DNA was extracted from 0.75–1.25 g (wet weight) by using the protocol described by Gevers *et al.* (2001), using a combination of glass beads and enzymes, but with the following modifications. Volumes were increased tenfold for application on a large scale. Vortexing with beads of the SDS-treated cells was performed for 30 s. After addition of and gentle shaking with, respectively, 16.5 ml buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl, the suspension was incubated at 65 °C for 10 min. Subsequent chloroform/isoamyl alcohol extraction, precipitation, spooling of DNA on a glass rod, washing with ethanol and RNase treatment were performed as described by Marmur (1961). For determination of the DNA base composition, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters SymmetryShield C8 column maintained at a temperature of 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The G+C content of DNA was 44 mol% for all three tempoyak strains and 45 mol% for *Leuconostoc fructosum*;

the latter value is close to the value of 43.4 mol% given by Antunes *et al.* (2002) for *Leuconostoc fructosum*.

DNA–DNA hybridizations were performed between strains LMG 22556^T, LMG 22557, LMG 22558 and *Leuconostoc fructosum* LMG 9498^T (DNA was prepared as described above). The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 39 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2.5% dextran sulphate, 50% formamide, 100 µg denatured salmon sperm DNA ml⁻¹, 1250 ng biotinylated probe DNA ml⁻¹). Hybridization levels between 97 and 100% were found between the three isolates; this indicates that the strains represent a single species. Values of 29–30% relatedness with *Leuconostoc fructosum* indicate that the tempoyak isolates represent a novel species.

Cell morphology and motility were tested by phase-contrast microscopy of cells grown in APT (All-Purpose-Tween; Difco) broth. The Gram reaction was performed as described by Gregersen (1978) and the catalase reaction was performed by addition of 3% H₂O₂ to colonies grown on APT agar. Two strains (LMG 22556^T and LMG 22558) were also tested for production of ammonia from arginine (Leisner *et al.*, 1994). Strain LMG 22556^T was tested for the following: ability to grow in APT broth incubated at 5, 15, 35 or 45 °C; growth in APT broth adjusted to pH 3.9; growth in APT broth with 2, 6.5, 8, 10 or 15% NaCl; growth on Rogosa agar (Oxoid) or STA agar (Oxoid); growth at pH reduced to below 4.15 in La broth [MRS broth lacking phosphate buffer with 0.3% (w/v) sodium citrate replacing ammonium citrate, and adjusted to an initial pH of 6.8 (Shaw & Harding, 1984)]; and reduction of nitrate during growth in Nutrient Broth (Oxoid) supplemented with 0.1% KNO₃. Strain LMG 22556^T was also tested for sensitivity

Fig. 1. Distance matrix tree showing the phylogenetic relationships of *Leuconostoc durionis* sp. nov. and other *Leuconostoc* reference species, based on 16S rRNA gene sequence comparisons. *Weissella confusa* was used as the outgroup and bootstrap probability values (percentages of 500 tree replications) are indicated at branch points.

during growth on soft APT agar (0.75%) plates to nisin produced by *Lactococcus lactis* ATCC 11454 and to pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0 by the deferred inhibition test (Carl *et al.*, 2004). Results of these morphological and biochemical tests are given below in the species description. Two strains (LMG 22556^T and LMG 22558) were tested (in Durham tubes) for gas production from 1% glucose and 1% gluconic acid in APT broth at 25 °C for 6 days. The three tempoyak isolates and the reference strain *Leuconostoc fructosum* LMG 9498^T were tested for gas and metabolite production in MRS broth at 30 °C for 24 h either with 1% D-glucose, xylose, ribose, lactose, sucrose or fructose as the sole carbohydrate source or with 1% glucose in combination with 1% fructose. All strains tested showed only limited growth with glucose, xylose, ribose, lactose, sucrose or fructose in MRS broth or with glucose or gluconic acid in APT broth. No gas production was detected from glucose in MRS broth or from glucose or gluconic acid in APT broth, but abundant gas production was observed by all strains in MRS broth with 1% glucose combined with 1% fructose, confirming that the three tempoyak strains are obligatorily heterofermentative. Analogous results were obtained for *Leuconostoc fructosum* LMG 9498^T. Antunes *et al.* (2002) had shown in a conventional test that fermentation of glucose by *Leuconostoc ficulneum* and *Leuconostoc fructosum* required addition of fructose. Sugar metabolites were analysed by HPLC or high-performance anion-exchange chromatography with pulsed amperometric detection as described by De Vuyst *et al.* (2002). Isomers of lactic acid were determined enzymically with a Boehringer kit. All three tempoyak strains produced, in addition to gas, the D-isomer of lactic acid (>97%) and acetic acid but not ethanol. When glucose and fructose were used as growth substrates, large amounts of mannitol were produced but erythritol was not detected (detection limit 10 mg l⁻¹).

Acid production from 49 carbohydrates or derivatives thereof was tested with strains LMG 22556^T, LMG 22557 and LMG 22558 using the API 50 CH kit according to the manufacturer's instructions (bioMérieux). The results are given in the species description below. The novel taxon is distinguished from the majority of other *Leuconostoc* species on the basis of no visible production of gas from glucose. *Leuconostoc fructosum* does not produce gas from glucose (this study); this is probably also the case for *Leuconostoc ficulneum*, but was not reported by Antunes *et al.* (2002). The taxon is distinguished from the two latter species based on its ability to produce acid from ribose, and from *Leuconostoc fructosum* also based on its ability to produce acid from sucrose, trehalose and D-turanose (Antunes *et al.*, 2002).

The results of the present study allowed us to assign strains LMG 22556^T, LMG 22557 and LMG 22558 to a novel species, for which we propose the name *Leuconostoc durionis* sp. nov.

Description of *Leuconostoc durionis* sp. nov.

Leuconostoc durionis (du.ri.o'nis. N.L. gen. n. *durionis* of *Durio*, the generic name of *Durio zibenthinus*, the durian fruit).

Surface colonies on APT agar after 3 days of micro-aerophilic incubation at 30 °C are <1–2 mm in diameter and round, with smooth surfaces and an off-white colour. Non-spore-forming rods occurring as single cells, as pairs or in chains. Cells are Gram-positive and non-motile in APT broth. Growth occurs at 5 and 35 °C but not at 45 °C. Growth occurs with 8.0% salt or at a pH of 3.9. Able to grow on Rogosa agar but not on STA agar. Unable to acidify pH <4.15 in La broth. Catalase-negative. Nitrate is not reduced. Ammonia is not produced from arginine. The D-lactic acid isomer is produced from glucose. Heterofermentative that produces lactic acid and acetic acid from D-glucose. Gas production is not detectable from glucose, but is abundant in combination with fructose. Not sensitive towards the nisin producer *Lactococcus lactis* ATCC 11454 or the pediocin PA-1 producer *P. acidilactici* PAC-1.0. Results for the API 50 CH kit summarized here are obtained after incubation for 5 days at 30 °C. Acid is produced from ribose, D-glucose, D-fructose, mannitol, sucrose, trehalose, D-turanose and gluconate. Negative results are obtained with glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside (LMG 22557 weak positive reaction), N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose (LMG 22557 weak positive reaction), lactose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2 ketogluconate and 5 ketogluconate. The G+C content of the DNA is 44 mol%.

The type strain, LMG 22556^T (=LAB 1679^T=D-24^T=CCUG 49949^T), and strains LMG 22557 (=LAB 1663=D-6) and LMG 22558 (=LAB 1674=D-18) were isolated from tempoyak, a Malaysian acid-fermented condiment made from pulp of the durian fruit.

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