Lactobacillus suntoryeus sp. nov., isolated from malt whisky distilleries

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Eight strains of *Lactobacillus* with identical partial 16S rRNA gene sequences and similar randomly amplified polymorphic DNA patterns were isolated from fermentation samples from Japanese and Scottish malt whisky distilleries. Phylogenetic analysis of almost complete 16S rRNA gene sequences from three representative strains (two from Japan, one from Scotland) placed them in the genus *Lactobacillus* as members of the *Lactobacillus acidophilus* group. *Lactobacillus helveticus* and *Lactobacillus gallinarum* were the most closely related species, with 16S rRNA gene similarities of 99.3 and 98.1 %, respectively. A similar phylogeny was derived from partial sequences of elongation factor Tu (*tuf*) genes in which the alleles from the three distillery isolates were identical and shared 99.0 % similarity with *L. helveticus* and *L. gallinarum tuf* genes. S-layer (*slp*) gene sequences suggested different relationships among the strains and the distillery isolates no longer formed a monophyletic group. The alleles from the Japanese and Scottish strains shared only 54 % similarity. Chromosomal DNA from the distillery strains gave DNA–DNA hybridization values between 79 and 100 % but showed less than 43 and 22 % reassociation with *L. helveticus* and *L. gallinarum* DNA, respectively. The name *Lactobacillus suntoryeus* sp. nov. is proposed for this novel taxon; the type strain is strain SA^T (=LMG 22464^T = NCIMB 14005^T).

Lactic acid bacteria comprise a natural component of the microflora of malt whisky fermentation (Simpson *et al.*, 2001; van Beek & Priest, 2002). In well-maintained distilleries their numbers are low at the beginning of the fermentation, but, once the yeast has completed the alcoholic fermentation, they grow prolifically during the 'late lactic fermentation' and are considered to confer positive flavour notes to the spirit (Takatani & Ikemoto, 2004). The most common species encountered are *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus paracasei*, but numerous other *Lactobacillus* species, lactococci, leuconostocs and weissellas have been detected, particularly in the early stages of the fermentation, when the alcohol concentration is relatively low (van Beek & Priest, 2003).

Analysis of Scotch whisky fermentations using denaturing gradient gel electrophoresis revealed an uncultured bacterium in the late stages of fermentation that was closely related to a bacterium isolated from a Japanese malt whisky fermentation and referred to as strain Y10. Phylogenetic

studies based on the 16S rRNA gene sequence of strain Y10 suggested that this bacterium was a member of the *Lactobacillus acidophilus* group (van Beek & Priest, 2002). This group comprises *L. acidophilus sensu stricto*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus* and *Lactobacillus johnsonii* (Johnson *et al.*, 1980; Fujisawa *et al.*, 1992; Gancheva *et al.*, 1999), with three relatively recent additions: *Lactobacillus amylolyticus*, isolated from malt and beer wort (Bohak *et al.*, 1998), *Lactobacillus iners*, associated with human sources (Falsen *et al.*, 1999) and *Lactobacillus kitasatonis*, from chicken intestines (Mukai *et al.*, 2003). In this study, we have investigated the phylogenetic relationships between the Japanese and Scottish isolates from whisky fermentations and shown that they constitute a distinct taxon within the *L. acidophilus* lineage, for which we propose the name *Lactobacillus suntoryeus* sp. nov.

Eight strains of lactic acid bacteria were isolated from four different distilleries in Japan and Scotland. The Japanese strains were isolated on three occasions: strains Y10, H8 and W6 in 1989, strains YD, HD and PD in 1993 and strain SA^T in 1999. Strains Y10, YD and SA^T were isolated from the Yamazaki distillery, strains H8, W6 and HD from the Hakushu distillery and PD from the Suntory pilot plant. Strain M4 was isolated from the Glenkinchie distillery in southern Scotland in 2002. For isolation of strain M4, a

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SA^T is AY445815.

RAPD profiles and a neighbour-joining tree based on partial *tuf* gene sequences are available as supplementary material in IJSEM Online.

fermentation sample was diluted in MRS broth, plated onto MRS agar (Oxoid) supplemented with maltose (10 g l^{-1}), brain heart infusion broth (Oxoid; 10 g l^{-1}) and cycloheximide ($100 \mu\text{g ml}^{-1}$) and incubated anaerobically for 48 h at 37°C . Isolated colonies were purified on the same medium by streak plating three times and a representative isolate was stored at -70°C in MRS broth containing 20 % (v/v) glycerol as strain M4. Distillery isolates were routinely cultured on supplemented MRS agar or broth (without cycloheximide) at 37°C anaerobically. The main reason why these organisms had not been recovered in earlier studies (e.g. Makanjuola & Springham, 1984; Simpson *et al.*, 2001) was probably due to their growth requirements; they grow poorly, if at all, unless MRS-based media are supplemented with brain heart infusion or similar. Reference strains *L. gallinarum* DSM 10532^T and *L. helveticus* NCIMB 11971^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and the National Collection of Industrial and Marine Bacteria (NCIMB), respectively, and cultured in MRS agar or broth at 37°C .

Chromosomal DNA was isolated using the PUREGENE DNA Isolation kit (Gentra Systems) from cultures grown in supplemented MRS broth for 24 h at 37°C . The manufacturer's protocol for DNA isolation from Gram-positive bacteria was modified by the addition of 140 U mutanolysin (Sigma) ml^{-1} and 50 μg lysozyme (Sigma) ml^{-1} to the lytic enzyme solution and the suspension was incubated at 37°C for 45 min to effect lysis. Larger amounts of DNA were prepared by phenol extraction as described previously (Simpson *et al.*, 2001). DNA concentration was determined by the diphenylamine method (Burton, 1968). Randomly amplified polymorphic DNA (RAPD) patterns were generated as described previously (Simpson *et al.*, 2001).

Fragments of genes for sequence determination were amplified by PCR using the following reaction mixture (50 μl): 5 μl DyNAzyme EXT buffer (Finnzymes), 1 μl 10 mM dNTPs, 2 μl 50 mM MgCl_2 , 10 pmol each primer, 100 ng DNA and 1 U DyNAzyme EXT polymerase. Amplifications were performed with the following thermal cycle: 95°C for 5 min, 30 cycles of 95°C for 10 s, an annealing step of 1 min (temperature specified below) and elongation at 72°C for 2 min, with a final elongation step at 72°C for 10 min. 16S rRNA genes were amplified using universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'), with an annealing temperature of 56°C . *tuf* genes were amplified using primers *tuf*-01-f (5'-GTTGTTGCTGCAACTGATGG-3') and *tuf*-02-r (5'-CCATTTCAGTACCTTCTGGC-3'), with an annealing temperature of 56°C . These were designed from an alignment of *tuf* gene sequences from *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum* and *L. helveticus* and enabled amplification of a region from nt 19 to 760 (numbering according to the *L. acidophilus* ATCC 4356^T gene sequence). S-layer genes were amplified using various primers with corresponding annealing

temperatures. The initial primers S01-f (5'-AGAATYGTAGCGYTGCTGC-3') and S02-r (5'-CGTTGTGCTTCAAGTACGC-3') at 50°C enabled partial sequencing of the 5' end of the PCR products from all four strains (three distillery isolates and *L. gallinarum* DSM 10532^T). New primers were then prepared for strains SA^T and Y10, S03-f (5'-GACCACATGAAGAAAAATTT-3') and Jap3-S04-r (5'-CAGCACCATCAACAGCAATA-3') at 50°C ; for strain M4, S01-f and M4-S04-r (5'-TCGTCAGCAGTAGTAGATGG-3') at 50°C ; and for *L. gallinarum* DSM 10532^T, S01-f and galli-S04-r (5'-GTTTCATAGCCTTAAGAGCTG-3') at 56°C , as conserved regions suitable for all strains were not available. This resulted in fragments of 848 bp (nt 49–896) for strains Y10 and SA^T, 711 bp (nt 70–780) for strain M4 and 808 bp (nt 70–877) for *L. gallinarum* DSM 10532^T all based on *L. acidophilus* ATCC 4356^T gene sequence numbering. PCR products were purified by PEG₈₀₀₀ precipitation and sequenced using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and the ABI 310 capillary sequencing system (Applied Biosystems). Due to the presence of multiple copies of the S-layer gene in *L. gallinarum* DSM 10532^T, PCR products were cloned in *Escherichia coli* with the TOPO TA Cloning kit (Invitrogen) and sequenced after PCR amplification with primers S01-f and galli-S04-r as described above. Alignments were prepared with CLUSTAL X and phylogenetic trees were constructed using PAUP 4 (<http://paup.csit.fsu.edu/>).

For DNA–DNA hybridizations, probe DNAs were labelled with digoxigenin using random-priming and the DIG High Prime DNA Labelling and Detection Starter kit II (Roche). DNA (1 μg) was immobilized on nylon membranes, hybridized at 42°C and washed at high stringency (65°C) as described previously (Simpson *et al.*, 2001). Labelled probe was detected by chemiluminescence and quantified with ImageMaster TotalLab software (Amersham Pharmacia Biotech).

Partial 16S rRNA gene sequences (around 300 bp from the 5' end) of the eight strains were identical. RAPD analysis provided further evidence that the distillery isolates were closely related. RAPD patterns are highly discriminatory for members of the *L. acidophilus* group and can be used to distinguish strains of the various species, as well as to provide discrimination within species (Du Plessis & Dicks, 1995; Gancheva *et al.*, 1999; Ventura & Zink, 2002). The identical patterns generated from strains SA^T and Y10 and the very similar pattern from strain M4 were consistent with the isolates belonging to a single species (see Supplementary Fig. A available in IJSEM Online).

The virtually complete 16S rRNA gene sequences of strains SA^T, Y10 and M4 and of *L. helveticus* NCIMB 11971^T were determined and a phylogenetic tree was constructed using 16S rRNA gene sequences retrieved from GenBank for other members of the *L. acidophilus* group and *L. brevis*. The three distillery isolates had identical 16S rRNA gene sequences and the tree topology confirmed that they were members of the *L. acidophilus* group (Fig. 1). The 16S rRNA

Fig. 1. Neighbour-joining tree showing the relationships of the distillery isolates with reference strains of the *L. acidophilus* group based on 16S rRNA gene sequences. *L. brevis* was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Sequences generated in this study are indicated in bold. Bar, 0.005 substitutions per site.

genes of these strains shared 99.3 and 98.1 % sequence similarity with *L. helveticus* and *L. gallinarum* 16S rRNA genes, respectively.

To differentiate further between the distillery isolates and their relatives, we analysed two protein-coding genes, the *tuf* gene, which encodes the translational elongation factor Tu (Chavagnat *et al.*, 2002) and *slp*, which encodes a surface-layer protein. A neighbour-joining tree based on partial sequences of 681 bp of the *tuf* genes fully supported the tree topology obtained with 16S rRNA gene sequences. The three distillery isolates had identical *tuf* alleles and were again most closely related to *L. helveticus* and *L. gallinarum* (see Supplementary Fig. B available in IJSEM Online), confirming the value of this gene as a reliable indicator of phylogenetic relationships within this group of bacteria (Chavagnat *et al.*, 2002).

Lactobacilli of the *L. acidophilus* group, apart from *L. johnsonii* and *L. gasseri*, possess a highly variable surface-layer protein (Boot *et al.*, 1996) that has been used as a taxonomic tool for identification of *L. crispatus* (Horie *et al.*, 2002). We amplified and sequenced partial *slp* genes from the three distillery isolates and *L. gallinarum* DSM 10532^T. The topology of the neighbour-joining tree constructed from these sequences and homologous sequences from GenBank (Fig. 2) differed from that based on 16S rRNA and *tuf* genes, and the distillery isolates did not form a monophyletic group. Indeed, the allele of strain M4 shared very low sequence similarity (approx. 54 %) with those of the Japanese strains. Surface-protein genes are subject to greater selective pressure and change more rapidly than housekeeping genes such as *tuf*, particularly in pathogenic bacteria exposed to immune responses

Fig. 2. Neighbour-joining tree showing the relationships of the distillery isolates with reference strains of the *L. acidophilus* group based on *slp* gene sequences. *L. brevis* was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Sequences generated in this study are indicated in bold. Bar, 0.1 substitutions per site.

(van Loo *et al.*, 2002). The inconsistencies between the *slp* tree and those derived from 16S rRNA and *tuf* genes indicated that the *slp* gene may not provide an accurate picture of the phylogenetic relationships of these organisms and may be subject to lateral transfer.

Since the 16S rRNA and *tuf* gene sequences showed that the distillery isolates were most closely related to *L. gallinarum* and *L. helveticus*, we established the status of these strains by DNA hybridization. Probe DNA from strains SAT and M4 gave DNA–DNA hybridization values between 79 and 100 % with each other and with DNA from strain Y10. Hybridization between the two probe DNAs and DNA from *L. gallinarum* DSM 10532^T and *L. helveticus* NCIMB 11971^T was less than 43 %, establishing the distillery isolates as representatives of a novel species.

The three distillery strains SAT, Y10 and M4 were all Gram-positive, catalase-negative homofermentative rods. After anaerobic incubation in modified MRS broth for 24 h, SAT cells were rods approximately 10 µm long (ranging from 2 to 30 µm), M4 cells were smaller (from 2 to 15 µm) and Y10 cells formed long filaments (from 20 to 200 µm). The carbohydrate fermentation patterns of the three distillery strains and *L. gallinarum* DSM 10532^T were determined using the API 50 CHL system (bioMérieux). The three distillery strains had similar profiles except for the production of acid from galactose and starch, which was positive for strain M4 and negative for strains SAT and Y10 (Table 1). The phylogenetic analyses, DNA hybridization results and distinguishing phenotypic characteristics (Table 1) confirmed these bacteria as members of a novel species, for which we propose the name *Lactobacillus suntoryeus* sp. nov. in recognition of the first isolation of these bacteria.

Description of *Lactobacillus suntoryeus* sp. nov.

Lactobacillus suntoryeus (sun.to.ry.e'us. N.L. masc. adj. *suntoryeus* occurring in Suntory malt whisky fermentations).

Table 1. Differential carbohydrate fermentation reactions of distillery isolates compared with related strains of the *L. acidophilus* group

Taxa: 1, *L. suntoryeus* sp. nov. strains SA^T and Y10; 2, *L. suntoryeus* sp. nov. M4; 3, *L. acidophilus*; 4, *L. crispatus*; 5, *L. gallinarum* DSM 10532^T; 6, *L. helveticus*. Data in columns 3, 4 and 6 adapted from API 50 CHL literature; other results determined in this study. Characters are scored as: +, 85–100 % positive; (+), 75–84 % positive; v, variable (26–74 % positive); (–), 16–25 % positive; –, 0–15 % positive. Results for the other fermentation reactions are given in the species description.

Acid from:	1	2	3	4	5	6
Cellobiose	+	+	+	+	+	–
Galactose	–	+	(+)	(+)	+	+
Gentiobiose	+	+	+	v	+	–
Glycogen	–	–	–	v	+	–
Raffinose	–	–	(–)	v	+	–
Starch	–	+	(–)	+	+	–
Trehalose	–	–	(+)	v	–	v

Gram-positive rods varying in length from 2 µm to long filaments. Non-motile, non-sporulating and catalase negative. After 48 h incubation on MRS agar (supplemented with maltose and brain heart infusion broth), colonies are circular, shiny, creamy white in colour and 2–5 mm in diameter. Growth is poor in unsupplemented MRS medium. Growth occurs at 37 and 45 °C, but not at 15 °C. Strictly homofermentative. Acid is produced from *N*-acetyl glucosamine, amygdalin, arbutin, cellobiose, aesculin, fructose, gentiobiose, glucose, maltose, mannose, salicin, sucrose and in some cases from galactose and starch (type strain negative). Adonitol, D-arabinose, L-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, gluconate, glycerol, inositol, inulin, 2-ketogluconate, 5-ketogluconate, D-lyxose, mannitol, melezitose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-xyloside, rhamnose, ribose, sorbitol, sorbose, D-tagatose, D-turanose, xylitol and D- and L-xylose are not fermented. Strains have been isolated from whisky fermentations.

The type strain is strain SA^T (=LMG 22464^T=NCIMB 14005^T).

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