Enterococcus saccharominimus sp. nov., from dairy products

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Four isolates, which were obtained from Belgian, Moroccan and Romanian dairy products, constituted a homogeneous but unidentified taxon after screening with whole-cell protein fingerprinting. Complete 16S rRNA gene sequence analysis classified representative strains in the genus *Enterococcus*. Highest sequence similarities of 98.6 and 98.0% were obtained with the species *Enterococcus sulfureus* and *Enterococcus saccharolyticus*, respectively. Growth characteristics, biochemical features, tRNA intergenic length polymorphism analysis, DNA–DNA hybridization and DNA G+C contents of selected strains demonstrated that they represent a single, novel *Enterococcus* species. It differs phenotypically from other enterococci in characteristics commonly considered as typical of this genus: no growth in 6.5% NaCl or 0.4% sodium azide, and no acid production from a wide range of carbohydrates. The name *Enterococcus saccharominimus* sp. nov. is proposed for this novel species; the type strain (LMG 21727^T = CCM 7220^T) was isolated from contaminated pasteurized cow's milk.

Identification techniques that are linked to extensive databases are helpful to discern novel groups of bacteria. For lactic acid bacteria (LAB), SDS-PAGE of whole-cell proteins has been intensively used as a routine identification system; the approach is linked to a database that comprises nearly all LAB species with validly published names (Pot *et al.*, 1994). Numerical analysis showed that four LAB strains, isolated from dairy sources from three different

countries, occupied a homogeneous but separate position in the dendrogram. In the present study, the taxonomic position of the four strains is investigated.

In an ongoing study, strains were isolated on different occasions from LAB in dairy products. One Belgian strain, LMG 21727^T, was obtained in 2002 from a dairy factory where the strain was isolated as a contaminant of pasteurized cow's milk on plate count agar (Oxoid) at 30 °C under aerobic conditions. Two Romanian strains, LMG 22195 (= R-19307) and LMG 22196 (= R-19653), were obtained in 2002 from a commercial fermented milk product and raw cow's milk, respectively. Strain LMG 22195 was isolated and purified after direct plating on de Man–Rogosa–Sharpe agar (MRS; Oxoid) and incubation at 37 °C under aerobic conditions. Strain LMG 22196 was isolated and purified on

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of LMG 21727^T, LMG 22195 and LMG 22197 are AJ626902, AJ626903 and AJ626904, respectively.

A figure showing an extended phylogenetic tree is available as supplementary material in IJSEM Online.

Table 1. Strains studied

Species	Other strain designation*	Source, place and year of isolation
<i>E. saccharominimus</i> LMG 21727 ^T	CCM 7220 ^T	Contaminant from pasteurized cow's milk, Belgium, 2002
LMG 22195	16.2, R-19307	Commercial fermented milk product, Romania, 2002
LMG 22196	56.9, R-19653	Raw cow's milk, Romania, 2002
LMG 22197	B5, R-7861, CCMM B208	White soft cheese, Morocco, 1999
<i>E. saccharolyticus</i> LMG 11427 ^T	NCFB 2594 ^T	Straw bedding
<i>E. sulfureus</i> LMG 13084 ^T	NCFB 2379 ^T	Plant material

*CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic; CCMM, Collections Coordonnées Marocaines de Microorganismes, Rabat, Morocco; LMG, BCCM/LMG Bacteria Collection Laboratorium voor Microbiologie, Ghent University, Gent, Belgium; NCFB, National Collection of Food Bacteria, Agricultural and Food Research Council (AFRC), Institute of Food Research, Aberdeen, Scotland, UK; R, Research Collection Laboratorium voor Microbiologie, Ghent University, Gent, Belgium.

Streptococcus thermophilus agar (Dave & Shah, 1996) and aerobic incubation at 42 °C. A Moroccan strain, LMG 22197 (= R-7861 = CCMM B208), was isolated in 1999 from white soft cheese after enrichment in MRS broth for 24 h at 30 °C under aerobic conditions and purification on MRS agar.

Basic microbiological tests demonstrated that the strains were Gram-positive, catalase- and oxidase-negative, non-motile cocci. Cultivation conditions for further experiments and for maintenance of the dairy isolates and reference strains listed in Table 1 were MRS agar and incubation at 30 °C for 24–48 h, unless indicated otherwise.

All four dairy isolates were investigated by using PAGE of whole-cell proteins. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of protein profiles and numerical analysis were performed by using the Gelcompar software package, versions 3.1 and 4.0, respectively (Applied Maths). Whole-cell protein profiles of the dairy isolates were initially compared with an in-house database, comprising profiles of nearly all LAB species with validly published names. The strains constituted a homogeneous and separate cluster

among profiles of enterococci (Pot & Janssens, 1993; data not shown). Fig. 1 shows a dendrogram that was obtained after UPGMA linkage cluster analysis of all four dairy isolates and the type strains of *Enterococcus saccharolyticus* and *Enterococcus sulfureus* (closest phylogenetic neighbours; see below).

The phylogenetic position of three representative strains, LMG 21727^T, LMG 22195 and LMG 22197, was determined by complete 16S rRNA gene sequence analysis. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). 16S rRNA gene amplification, purification and sequencing were performed as described by Vancanneyt *et al.* (2004) with the following modifications. PCR-amplified 16S rRNA genes were purified by using a NucleoFast 96 PCR clean-up kit (Macherey-Nagel). Sequencing reactions were performed by using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and purified by using a Montage SEQ₉₆ sequencing reaction cleanup kit (Millipore). Sequencing was performed by using an ABI Prism 3100 genetic analyser (Applied Biosystems). Sequence assembly was done by using the program AutoAssembler (Applied Biosystems). The 16S rRNA gene sequences (continuous stretches of 1509 bp) and sequences of strains retrieved from

Fig. 1. Protein profiles and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (*r*, expressed as a percentage value for convenience) of *E. saccharominimus* strains and some related reference species.

GenBank/EMBL were aligned, and a phylogenetic tree was constructed by using the neighbour-joining method using the Bionumerics software package, version 3.50 (Applied Maths). Unknown bases were discarded in 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. 2; an extended version of Fig. 2 can be viewed as supplementary material in IJSEM Online). The complete determined sequences of the three dairy isolates revealed similarities higher than 99.9%. Comparison with deposited sequences available in GenBank/EMBL classified the strains in the genus *Enterococcus* with nearest neighbours *E. sulfureus* and *E. saccharolyticus* (sequence similarities of 98.6 and 98.0%, respectively). The latter reference taxa and the dairy isolates constituted a separate branch in the phylogenetic tree. Lower sequence similarities (97.6–97.5%) were obtained with *Enterococcus gallinarum* and *Enterococcus casseliflavus*.

In tRNA intergenic length polymorphism analysis, performed as described previously (Baele *et al.*, 2000), the strains showed similar electrophoretic patterns differing from all described enterococcal species (data not shown).

DNA G+C contents were determined for three dairy isolates, LMG 21727^T, LMG 22195 and LMG 22197, and the reference strains *E. saccharolyticus* LMG 11427^T and *E. sulfureus* LMG 13084^T. Cells were cultivated in MRS broth at 37 °C for 24 h. DNA was extracted from 0.5–0.75 g (wet wt) by using the protocol described by Marmur (1961) with the following modifications: (i) cells were suspended overnight in Tris/HCl buffer pH 8.0 that contained lysozyme (8 mg ml⁻¹) before addition of SDS and (ii) lysed cells were treated with proteinase K (360 mg l⁻¹; Merck) at 37 °C for 2 h. For determination of the DNA G+C content, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture 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 λ -phage DNA (Sigma) was used as the calibration reference. DNA G+C contents of strains LMG 21727^T, LMG 22195 and LMG 22197 were 39, 39 and 38 mol%, respectively. These values are similar to the DNA G+C contents determined for the type strain of *E. saccharolyticus* LMG 11427^T (37 mol%) and of *E. sulfureus* LMG 13084^T (38 mol%), results which confirm the data described in literature (Devriese & Pot, 1995).

DNA–DNA hybridizations were performed between strains LMG 21727^T, LMG 22195 and LMG 22197, *E. saccharolyticus* LMG 11427^T and *E. sulfureus* LMG 13084^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 a HTS7000 bio assay reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 36 °C in hybridization mixture (2 $\times$ SSC, 5 $\times$ Denhardt's solution, 2.5% dextran sulfate, 50% formamide, 100 μ g denaturated salmon sperm DNA ml⁻¹, 1250 ng biotinylated probe DNA ml⁻¹). The DNA relatedness percentages presented are means, based on at least two independent hybridization experiments. Reciprocal reactions (e.g. A $\times$ B and B $\times$ A) were performed and also considered as independent hybridization experiments.

Hybridization levels of 11–13% were found between the type strain of *E. sulfureus* and strains LMG 21727^T, LMG 22195 and LMG 22197; and a level of 11% between the type strain of *E. saccharolyticus* and strains LMG 21727^T, LMG 22195 and LMG 22197. Hybridization levels of 72–79% were found between LMG 21727^T, LMG 22195 and LMG 22197; this indicates that the dairy strains constitute a single species.

Fig. 2. Distance matrix tree showing the phylogenetic relationships of *E. saccharominimus* and other enterococcal reference species, based on 16S rRNA gene sequence comparisons. *Enterococcus cecorum* was used as the outgroup and bootstrap probability values (percentages of 500 tree replications) are indicated at branch-points.

Growth tests were carried out as described by Švec *et al.* (2001). Acidifications of carbohydrates were recorded after 3 days incubation in API 50 CH galleries under paraffin cover. Lancefield antigens were detected using the Streptococcal grouping kit (Oxoid). Biochemical reactions were determined in the API 20 STREP system (bioMérieux). VP tests were duplicated using Voges–Proskauer diagnostic tablets (Rosco). Although the new taxon evidently belongs to the genus *Enterococcus*, several phenotypic traits do not conform with the physiological characteristics attributed to this genus. The strains did not tolerate 6·5 % NaCl and growth and aesculin degradation on bile aesculin agar (Oxoid) was poor. They were severely inhibited by 0·4 % sodium azide-containing Slanetz–Bartley agar (Oxoid). Moreover, they did not produce acid from carbohydrates such as ribose, β -gentiobiose and arbutin, which are commonly degraded by enterococci (Devriese *et al.*, 1993). A detailed description of other phenotypic characteristics of the novel species is given below and characteristics differentiating the novel species from its closest relatives *E. sulfureus* and *E. saccharolyticus* are shown in Table 2.

The overall results of the present study allowed us to assign the four strains LMG 21727^T, LMG 22195, LMG 22196 and LMG 22197 to a novel species, for which we propose the name *Enterococcus saccharominimus* sp. nov.

Description of *Enterococcus saccharominimus* sp. nov.

Enterococcus saccharominimus (sac.cha.ro.mi'ni.mus Gr. n. *sacchar* sugar; L. sup. adj. *minimus*; very least N.L. sup. adj. *saccharominimus* meaning that this organism differs from other enterococci in that it lyses only few sugars).

Cells are irregularly sized, Gram-positive and coccal or ovoidal, which are predominantly arranged in small groups. Colonies on Columbia sheep blood agar are smaller than commonly seen with the enterococci (up to 2 mm in

diameter), unpigmented, regular and translucent, surrounded by zones of semi-transparent α -haemolysis. The strains grow equally well at 30 and 37 °C. Addition of 5 % CO₂ does not enhance growth. They form deposits with clear supernatants in brain heart infusion broth (Oxoid) and react weakly with Lancefield group D antiserum (Streptococcal grouping kit; Oxoid). Not motile. All strains tested positive in API 20 STREP tests with Voges–Proskauer reagents and for pyrrolidonyl arylamidase and leucine arylamidase. Aesculin is degraded weakly. In API 50 CH galleries, acid is produced from galactose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, maltose, lactose and sucrose. Reactions are strain-dependent for acid production from amygdalin, cellobiose, mannitol, methyl α -D-glucoside, salicin, trehalose, D-turanose and D-tagatose. All strains tested negative for activity of hippurate, β -glucuronidase, β -galactosidase, α -galactosidase and alkaline phosphatase, and for acid production from glycerol, erythritol, DL-arabinose, ribose, DL-xylose, adonitol, methyl β -xyloside, L-sorbose, rhamnose, dulcitol, inositol, methyl α -D-mannoside, arbutin, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, β -gentiobiose, xylitol, D-lyxose, DL-fucose, DL-arabitol, gluconate and 2- and 5-ketogluconate. DNA G + C content is 38–39 mol%. Habitat: dairy products.

The type strain is LMG 21727^T (= CCM 7220^T), which was isolated from contaminated pasteurized cow's milk.

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Table 2. Characteristics that differentiate *E. saccharominimus* from its closest phylogenetic relatives

Species: 1, *E. saccharominimus*; 2, *E. sulfureus*; 3, *E. saccharolyticus*.

Characteristic	1	2	3
Resistance to:			
6·5 % NaCl	–	+	+
40 % bile	Weak	+	+
Group D antigen	+	–	–
α -Galactosidase	–	+	+
Acid from:			
Arbutin	–	+	+
β -Gentiobiose	–	+	+
2-Ketogluconate	–	+	+
Ribose	–	+	+

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