Enterococcus devriesei sp. nov., associated with animal sources

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The taxonomic position of two bovine strains, LMG 13603 and LMG 14595, assigned to the species *Enterococcus raffinosus* on the basis of biochemical features, was reinvestigated. Both reference strains and two other isolates, 6/1 (=LMG 22829) originating from a charcoal-broiled river lamprey and IE38.4 (=LMG 22830) from the air of a poultry slaughter by-product processing plant, occupied a clearly separate position, on the basis of sequence analysis of the housekeeping gene *pheS* (encoding the phenylalanyl-tRNA synthase α-subunit), relative to the type strain of *E. raffinosus* and all other enterococcal species with validly published names. 16S rRNA gene sequencing of strains LMG 13603, LMG 14595, 6/1 and IE38.4 confirmed their phylogenetic position in the *Enterococcus avium* species group, there being more than 99 % 16S rRNA gene sequence similarity to most members of the group, including *E. raffinosus*, and revealed *Enterococcus pseudoavium* as the closest phylogenetic relative (99·8–99·9 %). Further phenotypic and genotypic analyses using whole-cell-protein electrophoresis, $(GTG)_5$-PCR fingerprinting, ribotyping and DNA–DNA hybridization experiments demonstrated that all four strains represent a novel enterococcal species, for which the name *Enterococcus devriesei* sp. nov. is proposed. The type strain is LMG 14595^T (=CCM 7299^T).

In phylogenetic terms, the genus *Enterococcus* belongs to the clostridium branch of the Gram-positive bacteria. On the basis of 16S rRNA sequence analysis, the majority of enterococcal species can be classified in species groups, e.g. the *Enterococcus avium*, *Enterococcus cecorum*, *Enterococcus faecalis*, *Enterococcus faecium* or *Enterococcus gallinarum* species groups, or represent phylogenetically distinct lineages (Devriese & Pot, 1995; Williams *et al.*, 1991). Identification based on classical phenotypic tests is still valuable for the most common species or species groups, but for several newly described species molecular methods are required.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *E. devriesei* LMG 14595^T, LMG 13603, LMG 22829 and LMG 22830 are AJ891167, DQ010644, DQ010642 and DQ010643, respectively.

A distance matrix tree, $(GTG)_5$-PCR fingerprint patterns and ribotype patterns for *E. devriesei* sp. nov. and phylogenetically related enterococcal species are available as supplementary figures in IJSEM Online.

During the construction of a sequence-based identification approach for enterococci, representative strains of all enterococcal species with validly published names were investigated using sequence analysis of the housekeeping gene *pheS* (which encodes the phenylalanyl-tRNA synthase α-subunit). The type strain of *Enterococcus raffinosus* (LMG 12888^T) and three reference strains (LMG 12172, LMG 13603 and LMG 14595) assigned as *E. raffinosus* in the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium) were analysed and grouped into two separate branches. Strains LMG 13603 and LMG 14595, which are of bovine origin and were identified by means of biochemical characteristics, were clearly distinct from the type strain of *E. raffinosus* (LMG 12888^T) and LMG 12172, which are of human origin. At the same time, a screening study using ribotyping revealed that the *E. raffinosus*-like strains, LMG 13603 and LMG 14595, possessed patterns very different from that of *E. raffinosus* LMG 12888^T and very similar to those of two novel isolates, LMG 22829 and LMG 22830, originating from a charcoal-broiled river lamprey and the air of a poultry slaughter by-product processing plant (see below). The aberrant position of the latter four strains has led to a polyphasic study designed to elucidate their taxonomic positions.

Strains LMG 14595 (=CCM 7299) and LMG 13603 (=CCM 7298), both of which are of bovine origin, were isolated by L. A. Devriese (Belgium) and provided in 1994 and 1993, respectively. Two recent isolates were provided by the Department of Food and Environmental Hygiene, University of Helsinki, Finland: strain IE 38.4 (=LMG 22830) was isolated from the air of a poultry slaughter by-product processing plant, and strain 6/1 (=LMG 22829) was isolated from vacuum-packaged charcoal-broiled river lampreys. *E. raffinosus* LMG 12888^T and LMG 12172, both of which originate from human blood, were provided by R. Facklam (Centers for Disease Control, Atlanta, GA, USA) in 1992. All other enterococcal type strains included in this study were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/lmg.htm). All isolates were routinely grown on MRS agar plates. The plates were incubated under an anaerobic CO_2 atmosphere [Anaerogen (Oxoid); 9–13 % CO_2, according to the manufacturer's instructions] at 37 °C for 24 h.

After initial screening, by sequence analysis, of housekeeping gene *pheS* of LMG 13603 and LMG 14595 and subsequent comparison with all *Enterococcus* species with validly published names, the novel isolates (LMG 22829 and LMG 22830) were also tested. The *pheS* primers designed enabled the amplification, sequencing and comparison of a 455 bp fragment. Amplification and sequencing reactions were performed as described by Naser *et al.* (2005). The neighbour-joining (Saitou & Nei, 1987) tree of the *pheS* housekeeping gene investigated showed that strains LMG 13603, LMG 14595, LMG 22829 and LMG 22830 formed a homogeneous (>98·1 % similarity), but distinct, cluster. The type strain of *E. raffinosus* and strain LMG 12172, and other enterococcal species with validly published names, formed separate lineages (Fig. 1). The closest similarity was with *Enterococcus pseudoavium* (84·9 %). At the interspecies level, all enterococcal species could be clearly differentiated on the basis of *pheS* gene sequences, the maximum similarity being 86 %. Evaluation of the intraspecies variation showed that the *pheS* gene had a high degree of homogeneity among strains of the same species. Strains of the same enterococcal species had *pheS* gene sequence similarity of at least 97 % (Naser *et al.*, 2005).

Phylogenetic analysis based on complete 16S rRNA gene sequence determination was performed for LMG 14595, LMG 13603, LMG 22829 and LMG 22830 as described by Vancanneyt *et al.* (2004). The sequences obtained, and those of related species (downloaded from the GenBank database), were aligned using BioEdit software (Hall, 1999). Evolutionary distances were calculated using the Jukes–Cantor evolutionary model (Jukes & Cantor, 1969) and a phylogenetic tree was constructed using the neighbour-joining method with

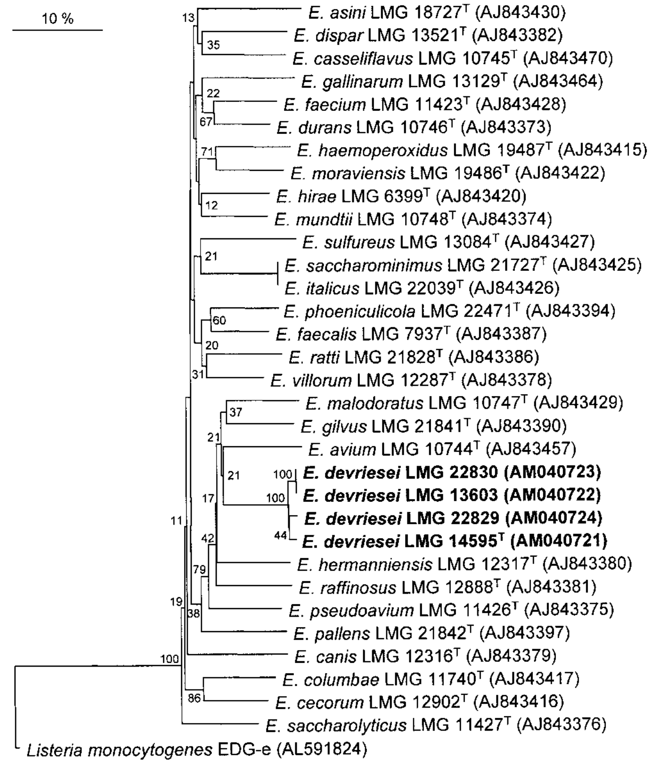

Fig. 1. Neighbour-joining tree based on the *pheS* gene sequences of *E. devriesei* sp. nov. and all enterococcal type strains. *Listeria monocytogenes* EGD-e was included as an out-group. Bootstrap values (from 500 tree simulations) are indicated at the branch points. Accession numbers are given in parentheses. Bar, 10 % evolutionary difference.

TREECON software (Van de Peer & de Wachter, 1994). The phylogenetic analysis placed strains LMG 14595, LMG 13603, LMG 22829 and LMG 22830 in a single cluster (99·8–100 % mutual sequence similarity) and assigned them as members of the *E. avium* species group. The highest levels of 16S rRNA gene sequence similarity were obtained with strains of *E. pseudoavium* (99·8–99·9 %), *E. raffinosus* (99·3 %), *Enterococcus gilvus* (99·3 %), *Enterococcus malodoratus* (99·4 %) and *E. avium* (99·2–99·3 %). *Enterococcus pallens* (98·2–98·3 %) and *Enterococcus hermanniensis* (97·2–97·3 %) were found to be more distantly related species within the *E. avium* species group. Supplementary Fig. S1 (available in IJSEM Online) shows the phylogenetic relationships of strains LMG 14595, LMG 13603, LMG 22829 and LMG 22830 with respect to members of the *E. avium* group and other representative enterococcal species. The results obtained confirmed the known high levels of 16S rRNA gene sequence similarity between individual members of the *E. avium* species group, as is also observed in, for example, the *E. gallinarum* (99·8 % similarity) or *E. faecium* species groups (99·7 % similarity) (Williams *et al.*, 1991). The housekeeping gene *pheS* proved to be a more discriminatory identification tool. The topology obtained in the *pheS* dendrogram, however, does not reflect the phylogenetic relationships revealed by 16S rRNA gene sequence analysis (Fig. 1, Supplementary Fig. S1).

SDS-PAGE analysis of whole-cell proteins extracted from cells grown for 24 h on MRS agar at 37 °C was performed in accordance with the procedure described by Pot *et al.* (1994). Protein profiles were compared with those in a BCCM/LMG Bacteria Collection database covering all enterococcal species with validly published names. The similarity between all pairs of traces was expressed by using the Pearson product-moment correlation coefficient converted, for convenience, to a percentage value. UPGMA (unweighted pair group method using arithmetic averages) clustering was used for construction of the dendrogram. Strain-specific differences were observed among the isolates, i.e. variable positions for dominant protein bands with a molecular mass of about 50 kDa. These dense bands strongly influenced the numerical analysis and it was only after omission of this variable region from the cluster analysis that the novel isolates occupied a distinct subgroup separate from other enterococcal species. The dendrogram (Fig. 2) shows the protein profiles obtained from the analysed strains and demonstrates their differentiation from the phylogenetically closest species accommodated in the *E. avium* species group.

Repetitive element primer-PCR fingerprinting with the $(GTG)_5$ primer and subsequent analysis of the patterns were performed using BioNumerics (version 4.0) software, as described by Gevers *et al.* (2001). The $(GTG)_5$-PCR fingerprints obtained were compared with those in the database (covering all enterococcal species with validly published names). Strains LMG 14595, LMG 13603, LMG 22829 and LMG 22830 had fingerprints that were visually similar. Cluster analysis grouped them into a single cluster clearly separated from the fingerprints obtained from the other enterococcal species. Supplementary Fig. S2 (available in IJSEM Online) shows the $(GTG)_5$-PCR fingerprint patterns obtained from the investigated strains and their differentiation from the type strains of the phylogenetically closest species accommodated in the *E. avium* species group.

For ribotyping, cells were grown at 25 °C either overnight in MRS broth or for 3 days on MRS agar plates. Chromosomal DNA was isolated as previously described by Björkroth & Korkeala (1996). *Hin*dIII and *Eco*RI enzymes were used for digestion of DNA, as specified by the manufacturer (New England Biolabs). Restriction enzyme analysis was performed as described previously (Björkroth & Korkeala, 1996) and Southern blotting was achieved using a vacuum device (Vacugene). The rDNA probe for ribotyping was labelled by reverse transcription [AMV-RT (Promega) and Dig labelling kit (Roche Molecular Biochemicals)] as described by Blumberg *et al.* (1991). Membranes were hybridized at 58 °C overnight and detection of the digoxigenin label was performed as recommended by Roche Molecular Biochemicals. Scanned (Scan Jet 4c/T; Hewlett Packard) ribopatterns were analysed using the BioNumerics version 3.5 software package. The similarity between all pairs of traces was expressed using the Dice correlation coefficient, and UPGMA clustering was used for the construction of the dendrograms. With reference to internal controls, a position tolerance of 1·5 % was allowed for the bands. The ribopatterns were compared with the corresponding patterns in the lactic acid bacteria database at the Department of Food and Environmental Hygiene, University of Helsinki, Finland. It contains the patterns for all relevant meat-associated lactic acid bacteria in the genera *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth & Korkeala, 1996, 1997; Björkroth *et al.*, 1998, 2000; Lyhs *et al.*, 1999). In the numerical analysis of the *Hin*dIII patterns [see Supplementary Fig. S3(a) available in IJSEM Online], strains LMG 14595, LMG 13603, LMG 22829 and LMG 22830 formed a cluster sharing pattern similarity of at least 79·9 %. The corresponding similarity between the *Eco*RI ribopatterns (Supplementary Fig. S3b) of the four strains was 84·2 %. *E. pallens* LMG 21842^T and *E. malodoratus* LMG 10747^T possessed patterns showing the highest levels of similarity (73·5 and 76·2 %) to the *Hin*dIII and *Eco*RI ribotypes, respectively, of the four strains. The dendrogram obtained by combining the unweighted pattern information for both the *Hin*dIII and *Eco*RI ribotypes into one numerical analysis is available as a supplementary figure in IJSEM Online (Supplementary Fig. S3c).

The DNA G+C content was determined and DNA–DNA hybridizations performed between strains LMG 14595, LMG 22829 and LMG 22830 and the following strains

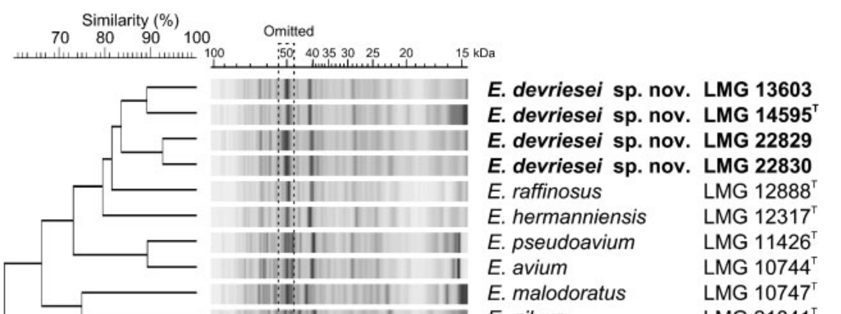

Fig. 2. Protein profiles of *E. devriesei* sp. nov. strains LMG 14595^T, LMG 13603, LMG 22829, LMG 22830 and the type strains of phylogenetically related species. The dendrogram was constructed by the UPGMA linkage of correlation coefficients (*r*, expressed, for convenience, as percentage-similarity values).

Table 1. Characteristics that differentiate *E. devriesei* sp. nov. from other species of the *E. avium* group

Taxa: 1, strains LMG 14595, LMG 13603, LMG 22829 and LMG 22830 (*E. devriesei* sp. nov.); 2, *E. hermanniensis*; 3, *E. avium*; 4, *E. pseudoavium*; 5, *E. raffinosus*; 6, *E. malodoratus*; 7, *E. gilvus*; 8, *E. pallens*. Characters are scored as follows: +, positive with rare negative exceptions; −, negative with rare positive exceptions. ND, Not determined. Characteristics are based on those obtained in the present study and/or those presented elsewhere (Devriese *et al.*, 1993; Tyrrell *et al.*, 2002).

Characteristic	1	2	3	4	5	6	7	8
Acid produced from:								
Lactose	+	−	+	+	+	+	+	+
Melibiose	−	−	−	−	+	+	+	+
2-Ketogluconate	−	−	+	+	+	+	ND	ND

representing the phylogenetically closest neighbours: *E. avium* LMG 10744^T, *E. raffinosus* LMG 12888^T, *E. malodoratus* LMG 10747^T, *E. pseudoavium* LMG 11426^T and *E. gilvus* LMG 21841^T. High-molecular-mass DNA isolation from bacterial cells grown on Todd–Hewitt broth (Oxoid), DNA G+C content determination and DNA–DNA hybridization (at a hybridization temperature of 33 °C) were performed as described by Vancanneyt *et al.* (2004). The hybridization temperature was calculated from the G+C content by using the formula of De Ley (1970), and was corrected for the presence of 50 % formamide in the hybridization mixture (McConaughy *et al.*, 1969). The DNA G+C content was 40 mol% for strains LMG 14595, LMG 22829 and LMG 22830. DNA–DNA hybridization values obtained between strains LMG 14595, LMG 22829 and LMG 22830 ranged from 68 ± 1 to 79 ± 9 %. Hybridization levels of 19 ± 1 to 32 ± 8 % were found between strains LMG 14595, LMG 22829 and LMG 22830 and *E. avium* species group type strains, including *E. raffinosus*. Similarly, DNA–DNA hybridization values in the range 25 ± 6 to 34 ± 4 % were found between the other type strains included in the experiment. These results confirmed that strains LMG 14595, LMG 22829 and LMG 22830 are members of a single novel species belonging to the *E. avium* species group.

For the determination of classical phenotypic properties, all strains were cultivated at 25 °C either overnight in MRS broth or for 3 days on MRS agar plates. Growth at different temperatures (2, 4, 10, 37 and 45 °C) or in the presence of NaCl (2, 4, 6·5, 8 and 10 %, w/v) was tested in MRS broth incubated until growth was observed or, otherwise, for at least 21 days. Isolates were tested for their carbohydrate-fermentation profiles by using the API 50 CH system (bioMérieux) and for biochemical activities by using the API STREP system (bioMérieux), in each case according to manufacturer's instructions. The production of ammonia from arginine was tested for in broth containing 0·5 % arginine, 0·5 % peptone, 0·3 % yeast extract, 0·1 % glucose and 0·016 % bromcresol purple. The formation of colonies typical of enterococci was tested for on bile–aesculin (Gibco) agar and Slanetz–Bartley (Oxoid) agar. Aerobic growth and haemolysis were tested for on bovine blood agar (Difco). Each test was carried out at least twice.

Phenotypic tests mainly resulted in reactions typical of enterococci, and, specifically, the *E. avium* species group, as listed by Devriese *et al.* (1993) and Devriese & Pot (1995). There were a few exceptions: unlike typical enterococci, none of the isolates grew at 45 °C or produced acid from methyl α-D-glucoside. The results are given in the species description below. Table 1 shows characteristics useful for differentiating the newly delineated taxon from the other species in the *E. avium* group.

All of the data obtained in the present study allowed us to assign strains LMG 14595, LMG 13603, LMG 22829 and LMG 22830 to a novel species, for which we propose the name *Enterococcus devriesei* sp. nov.

Description of *Enterococcus devriesei* sp. nov.

Enterococcus devriesei (de'vrie.se.i. N.L. gen. n. *devriesei* of Devriese, in honour of the Belgian microbiologist Luc A. Devriese for his outstanding contributions to the taxonomy of enterococci).

Cells are Gram-positive, catalase-negative, facultatively anaerobic cocci. Colonies on blood or MRS agar are white to light grey and translucent. On bovine blood agar there is α-haemolysis. Strains grow on azide-containing enterococcal selective agar as light- to dark-maroon colonies and cause blackening of bile–aesculin agar. Strains grow well at 10 and 37 °C. At 4 °C, growth can be somewhat slow; at 2 °C, growth does not occur. No growth is observed at 42 or 45 °C. All strains grow well in the presence of 2, 4 or 6·5 % NaCl. Strain 6/1 also grows in the presence of 8 % NaCl but not at 10 %. Strains react positively in the Voges–Proskauer test and negatively in tests for alkaline phosphatase, arginine dihydrolase, α- and β-galactosidase, β-glucuronidase and hippurate. Reactions in the tests for leucine arylamidase and pyrrolidonyl arylamidase are strain-dependent. Acid is produced from amygdalin, D-arabitol, arbutin, β-gentiobiose, cellobiose, D-fructose, D-glucose, D-mannose, galactose, lactose, maltose, mannitol, *N*-acetylglucosamine, ribose, sucrose, salicin and trehalose; all these reactions may be weak or delayed for strain LMG 22830, and reactions with amygdalin, D-arabitol, galactose and trehalose may be weak or delayed for strain LMG 14595^T. No acid is produced from starch, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-xyloside, D-fucose, L-fucose, dulcitol, erythritol, glycogen, inositol, inulin, melibiose, D-turanose, xylitol, D-xylose, L-xylose, 2-ketogluconate or 5-ketogluconate. Reactions with adonitol, gluconate, glycerol, melezitose, rhamnose, sorbitol, D-arabinose, L-arabinose, L-arabitol, D-lyxose, D-raffinose, D-tagatose and L-sorbose are strain-dependent (see Table 2). The G+C content of the DNA is 40 mol%.

Table 2. Strain-dependent characteristics for *E. devriesei* sp. nov.

Results were obtained in the present study, and are scored as described in Table 1 with the following additions; W, positive, but the reaction is weak and/or delayed.

Characteristic	LMG 14595^T	LMG 13603	LMG 22829	LMG 22830
Acid produced from:				
Adonitol	−	−	+	−
D-Arabinose	−	−	+	−
L-Arabinose	−	−	+	−
L-Arabitol	W	W	−	W
Gluconate	W	W	+	W
Glycerol	W	W	+	W
Lyxose	−	W	+	W
Melezitose	+	−	+	−
Raffinose	−	+	−	W
Rhamnose	W	W	W	−
Sorbitol	+	−	−	−
Sorbose	W	+	+	W
Tagatose	−	+	−	W
Pyrrolidonyl arylamidase	−	+	−	+
Leucine arylamidase	−	W	+	−

The type strain, which was isolated from bovine material, is LMG 14595^T (=CCM 7299^T). Reference strains are LMG 13603 (=CCM 7298), 6/1 (=LMG 22829) and IE38.4 (=LMG 22830).

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