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Enterococcus hermanniensis sp. nov., from modified-atmosphere-packaged broiler meat and canine tonsils

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Isolates 302, 334, 356, 377 and 379, detected in modified-atmosphere-packaged broiler meat, together with strains LMG 12317T and LMG 13617, detected in dog tonsils, were analysed in a polyphasic taxonomy study, including numerical analysis of ribopatterns and whole-cell protein patterns, 16S rRNA gene sequence analysis, DNA–DNA hybridization and determination of some phenotypic properties. The results indicated that these isolates represent a novel species in the genus Enterococcus. The isolates showed classical phenotypic reactions for the genus Enterococcus with the exception of not possessing the Lancefield group D antigen. Isolates 334, LMG 12317T and LMG 13617 showed the highest 16S rRNA gene sequence similarity (98.3–99.0%) to the Enterococcus pallens type strain. In the distance matrix tree based on 16S rRNA gene sequences, the three isolates were located in the Enterococcus avium group with E. pallens as their closest phylogenetic neighbour. Numerical analyses of whole-cell protein patterns and HindIII/EcoRI ribotypes placed all seven isolates together in a single cluster separated from the E. avium group reference strains. The DNA–DNA hybridization level between strains 334 and LMG 12317T was 93.5%, confirming that they represent the same species. Low hybridization levels (12–30%) were, by contrast, obtained with the E. pallens and Enterococcus raffinosus type strains. The name Enterococcus hermanniensis sp. nov. is proposed, with strain LMG 12317T (=CCUG 48100T) as the type strain.

Phylogenetically, the genus Enterococcus belongs to the clostridial branch of the Gram-positive bacteria. It was not officially recognized as a separate genus until 1984, when [Streptococcus] faecalis and [Streptococcus] faecium were reclassified as Enterococcus faecalis and Enterococcus faecium, respectively (Schleifer & Kilpper-Bälz, 1984). Since then, the number of enterococcal species described has increased, not only by the species transferred from the group D Streptococcus, but also by the description of several novel species. At the time of writing, over 20 species are classified as enterococci. Based on 16S rRNA gene sequence analyses, four phylogenetic groups (E. faecium, Enterococcus avium, Enterococcus gallinarum and Enterococcus cecorum species groups) have been described (Williams et al., 1991).

During a study of lactic acid bacteria (LAB) isolated from the spoilage population in modified-atmosphere-packaged (MAP), marinated, broiler legs, several unidentified LAB isolates were detected (Björkroth et al., 2004). Five isolates (302, 334, 356, 377 and 379) originating from fresh packages showed HindIII ribopatterns resembling the patterns of some known enterococci. The patterns of these five isolates were designated types UIVa and UIVb by Björkroth et al. (2004). When these isolates were initially studied by means of whole-cell protein analysis, they were found to possess similar patterns to two isolates (LMG 12317T and LMG 13617) originating from canine tonsils. The present polyphasic taxonomic study, including numerical analysis of ribopatterns and whole-cell protein patterns, 16S rRNA gene analysis, DNA–DNA hybridization and examination of essential phenotypic properties, set out to evaluate whether these seven isolates represent a novel enterococcal species.
All isolates were routinely cultured at 30 °C either overnight in MRS broth (Difco) or for 3 days on MRS agar plates (Oxoid). The plates were incubated under an anaerobic CO₂ atmosphere [Anaerogen (Oxoid); 9–13 % CO₂ according to the manufacturer] at 30 °C. For PAGE of whole-cell proteins, isolates were grown for 24 h on MRS agar (Oxoid) at 24 °C in a microaerobic atmosphere (approximately 5 % O₂, 10 % CO₂, 85 % N₂). All isolates were maintained in MRS broth (Difco) at −70 °C.

DNA for all analyses was isolated as described by Björkroth & Korkeala (1996). *HindIII* and EcoRI enzymes were used for digestion of DNA as specified by the manufacturer (New England Biolabs). Restriction endonuclease analysis was performed as described by Björkroth & Korkeala (1996) and Southern blotting was via a vacuum device (Vacugene; Pharmacia). The rRNA gene 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 (Hewlett Packard Scan Jet 4c/T) protein patterns were scanned using the BIONUMERICS 3.0 software package. The similarity between all pairs was expressed by the Dice coefficient correlation and UPGMA clustering was used for the construction of dendrograms. Based on the use of internal controls, a position tolerance of 1·5 % was allowed for the bands. The *EcoRI* and *HindIII* ribopatterns were compared to a previously established 16S RFLP patterns (ribotypes) of all *Enterococcus hermanniensis* sp. nov. strains examined and of *Enterococcus* type strains generated by *EcoRI* (a) and *HindIII* (b). Numerical analyses of the patterns are presented as dendrograms: left-hand side of the *EcoRI* and *HindIII* banding patterns possesses high molecular masses, <23 kbp, and right-hand side, >1000 bp.
were run in a Global IR2 sequencing device with E-SEQ 1.1 software (LiCor) according to the manufacturer's instructions. The consensus sequences of these isolates and representative strains belonging to the same phylogenetic group [retrieved from GenBank (http://www.ncbi.nlm.nih.gov), using BLASTN 2.2.6; Altschul et al., 1997] were aligned and a phylogenetic tree (Fig. 2) was constructed from the global alignment by the neighbour-joining algorithm using BIOPHARMERS 3.0 software package (Applied Maths). In the BLAST analyses, isolates LMG 12317T, LMG 13617 and 334 showed the highest 16S rRNA gene sequence similarities to 16S rRNA gene sequences of strains classified as Enterococcus pallels (98-3, 99-0, 98-9 %, respectively), Enterococcus pseudoavium (97-7, 98-4 and 98-2 %, respectively) and E. avium (97-7, 98-1 and 98-1 %, respectively). In the distance matrix tree based on 16S rRNA gene sequences (Fig. 2), the three isolates were located in the E. avium group with E. pallels as their closest phylogenetic neighbour.

For determination of the G + C content, DNA was enzymically degraded into nucleosides as described by Mesbah et al. (1989). The nucleoside mixture was then separated by HPLC using a Waters SymmetryShield C8 column thermostatted at 37 °C. The solvent was 0-02 M NH4H2PO4 with 1-5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki et al. (1989), using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 35 °C in 50 % formamide. The G + C content of DNA of isolates LMG 12317T and 334 was 37-1 and 36-6 mol%, respectively. The level of DNA–DNA relatedness between isolates 334 and LMG 12317T was 93-5 %, whereas the levels between isolate LMG 12317T and E. pallels or Enterococcus raffinosus type strains were only 11-5 and 23-7 %, respectively. Each value given is the mean of at least two hybridization experiments.

All isolates were Gram stained and tested for catalase. For size and precise morphology determinations in a transmission electron microscope, cells were suspended in physiological NaCl, negatively stained with 1 % phosphotungstic acid and examined using a JEOL JEM 100 electron microscope. A transmission electron micrograph of cells of strain LMG 12317T is available as Supplementary Fig. C in IJSEM Online. Growth at different temperatures (4, 37 and 45 °C) or in the presence of NaCl (2, 4, 6-5 and 10 % w/v) was tested in MRS broth (Difco) incubated until growth was observed or otherwise at least for 21 days. Lancefield antigen D was tested using the Streptococcal grouping kit (Oxoid). Isolates were tested for their carbohydrate fermentation profiles by API 50 CHL (bioMérieux) and for biochemical activities by API STREP identification systems (bioMérieux) according to the manufacturer’s instructions. Production of ammonia from arginine was tested in broth containing 0-5 % arginine, 0-5 % peptone, 0-3 % yeast extract, 0-1 % glucose and 0-016 % brom cresol purple. Formation of typical colonies for enterococci was tested on bile-esculin (Gibco) and Slanetz-Bartley (Oxoid) agars. Haemolyses were tested on blood agar. Each test was carried out at least twice. Phenotypic tests mainly resulted in typical reactions for enterococci as listed by Devriese et al. (1993) and Devrie & Pot (1995). There were some exceptions; all isolates were negative for Lancefield D, none grew at 45 °C, and isolates 302, 377 and 379 did not grow in the presence of 6-5 % NaCl. Detailed results are given in the species description below. Table 1 shows characteristics useful in differentiating Enterococcus hermanniensis sp. nov. from other species in the E. avium group.

On the basis of this polyphasic study, it is evident that isolates 302, 334, 356, 377, 379, LMG 12317T and LMG 13617 belong to a novel Enterococcus species within the E. avium group, for which we propose the name Enterococcus hermanniensis sp. nov. To date, E. hermanniensis has been isolated from MAP, fresh broiler leg packages and canine

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**Fig. 2.** Phylogenetic tree based on similarities of almost entire 16S rRNA gene sequences (at least 1400 bp) of Enterococcus hermanniensis sp. nov. and its phylogenetic neighbours. Bootstrap probability values from 500 trees resampled are given at the branch points.
Table 1. Characteristics that differentiate Enterococcus hermanniensis sp. nov. from the other species of the E. avium group

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<th>7</th>
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<td>+</td>
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<td>Acid production from:</td>
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Species: 1, E. hermanniensis sp. nov.; 2, E. avium; 3, E. pseudoavium; 4, E. raffinosus; 5, E. malodoratus; 6, E. glus; 7, E. gallinarum. Characters are scored as: +, strains positive with rare negative exceptions; –, strains negative with rare positive exceptions; D, strains may either be positive or negative; ND, not determined. Characters are based on those obtained in the present study and/or reported elsewhere (Devriese et al., 1993; Tyrrell et al., 2002).

Description of Enterococcus hermanniensis sp. nov.

Enterococcus hermanniensis (her.man.ni.en’sis. N.L. masc. adj. hermanniensis pertaining to Hermanni, a locality in Helsinki, Finland).

Strains are Gram-positive, catalase-negative, spherical to semispherical cocci growing in pairs (Supplementary Fig. C). Cells vary between 0·62 and 0·78 μm in width and 0·91 and 1·0 μm in length. Colonies on blood or MRS agar are 1–2 mm in diameter after 5 days of incubation, white to light grey and translucent. On bovine blood agar there is x-haemolysis. Strains grow slowly on azide-containing enterococcal selective media as typical, maroon colonies and cause blackening of bile-aeculin agar. Strains grow well at 10 and 37 °C but no growth is observed at 45 °C. All strains grow well in the presence of 2 or 4 % NaCl. Some strains (not strains 302, 377 and 379) grow in the presence of 6·5 % NaCl but none of the strains grows in the presence of 10 % NaCl. All strains react positively in Voges–Proskauer, pyrrolidonyl arylamidase and leucine arylamidase tests. Aesculin hydrolysis may be weak or delayed. Strains are negative in tests for Lancefield D antigen, hippurate hydrolysis and x-galactosidase, β-galactosidase, β-glucuronidase, alkaline phosphatase and arginine dihydrolase activity. As is typical for enterococci, acid is produced from arbutin, cellobiose, D-fructose, D-glucose, maltose, D-mannose, N-acetylglucosamine, ribose and salicin but not from erythritol, L-xylitol, glycerogen or fucose. Unlike most of the other enterococci, no acid is produced from lactose, galactose and amygdalin, although there may sometimes be a weak positive reaction with the latter two. All strains produce acid from mannitol. Acid production from D-arabitol, β-gentiobiose and trehalose may be weak or delayed. No acid production from adonitol, starch, L-arabinose, L-arabitol, dulcitol, ketogluconate, gluconate, glycerol, inositol, inulin, lyxose, melezitose, melibiose, methyl β-xylidade, D-raffinose, sucrose, sorbitol, L-sorbitose, tagatose, D-turanose, xylitol or D-xylitol is detected. Acid production from rhamnose and D-arabinose is weak or absent. The G+C content of DNA is 36–6–37·1 mol%.

The type strain is LMG 12317T (=CCUG 48100T), which was isolated from dog tonsils. Strains LMG 13617 and 334 (=LMG 21990) have also been deposited in the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG).

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