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

Joanna Koort,<sup>1</sup> Tom Coenye,<sup>2</sup> Peter Vandamme,<sup>2</sup> Antti Sukura<sup>3</sup> and Johanna Björkroth<sup>1</sup>

### Correspondence

Joanna Koort  
joanna.koort@helsinki.fi

<sup>1</sup>Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland

<sup>2</sup>Laboratory of Microbiology, University of Gent, Gent, Belgium

<sup>3</sup>Department of Basic Veterinary Sciences, University of Helsinki, Helsinki, Finland

Isolates 302, 334, 356, 377 and 379, detected in modified-atmosphere-packaged broiler meat, together with strains LMG 12317<sup>T</sup> 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 12317<sup>T</sup> 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 12317<sup>T</sup> 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 12317<sup>T</sup> (= CCUG 48100<sup>T</sup>) 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 12317<sup>T</sup> 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.

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**Abbreviations:** LAB, lactic acid bacteria; MAP, modified-atmosphere-packaged.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates 334, LMG 12317<sup>T</sup> and LMG 13617 are respectively AY396046, AY396047 and AY396048.

Dendrograms from combined ribotype analysis and whole-cell protein profiles and a micrograph of cells of strain LMG 12317<sup>T</sup> are available as supplementary material in IJSEM Online.

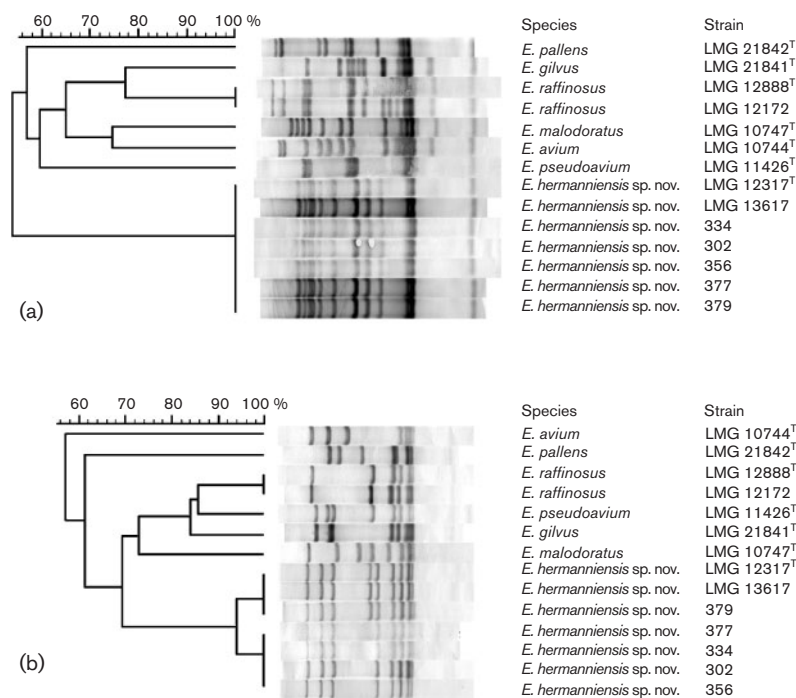
All isolates were routinely cultured at 30 °C either overnight in MRS broth (Difco) or for 3 days on MRS agar plates (Oxoid). The plates were incubated under an anaerobic CO<sub>2</sub> atmosphere [Anaerogen (Oxoid); 9–13 % CO<sub>2</sub> according to the manufacturer] at 30 °C. For PAGE of whole-cell proteins, isolates were grown for 24 h on MRS agar (Oxoid) at 24 °C in a microaerobic atmosphere (approximately 5 % O<sub>2</sub>, 10 % CO<sub>2</sub>, 85 % N<sub>2</sub>). All isolates were maintained in MRS broth (Difco) at –70 °C.

DNA for all analyses was isolated as described by Björkroth & Korkeala (1996). *Hind*III and *Eco*RI 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) ribopatterns were analysed 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 *Eco*RI and *Hind*III ribopatterns were compared to a previously established 16S and 23S rRNA gene RFLP LAB database. Dendrograms and banding patterns based on *Eco*RI and *Hind*III ribotypes are shown in Fig. 1(a) and (b), respectively. The dendrogram

obtained by combining the unweighted pattern information of both *Eco*RI and *Hind*III ribotypes into one numerical analysis is available as Supplementary Fig. A in IJSEM Online. In all RFLP analyses, the seven isolates formed tight and distinct clusters with sequence similarity values ranging from 94.1 to 100 %, whereas the similarity values between the patterns of these isolates and the reference strains located in the most adjacent clusters ranged from 56.2 to 69.4 %.

Preparation of cellular protein extracts and PAGE was performed as described by Pot *et al.* (1994). Whole-cell protein profiles were scanned using an LKB 2202 UltroScan laser densitometer. Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis using the Pearson product moment correlation coefficient were performed using the GELCOMP 4.2 software package (Applied Maths). The isolates investigated formed a homogeneous group clearly separated from reference strains of their closest phylogenetic neighbours (see below) (results from the numerical analysis of whole-cell protein patterns are available as Supplementary Fig. B in IJSEM Online).

The nearly complete 16S rRNA gene was amplified by PCR with a universal primer pair F8–27 (5'-AGAGTTTGATCCTGGCTGAG-3') and R1541–1522 (5'-AAGGAGGTGATCCAGCCGCA-3'). Sequencing of the purified (QIAquick PCR purification kit; Qiagen) PCR product was performed by Sanger's dideoxynucleotide chain-termination method using two long [primers F19–38 (5'-CTGGCTCAGGAYGAACGCTG-3') and R1541–1522] and two shorter reactions [primers F926 (5'-AACTCAAAGGAATTGACGG-3') and R519 (5'-GTATTACCGCGGCTGCTG-3')]. Samples



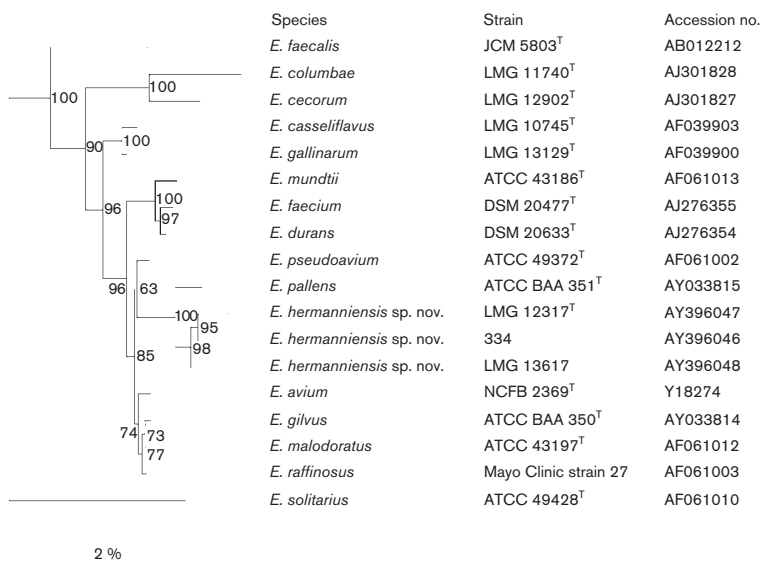
**Fig. 1.** Numerical analysis of 16 and 23S RFLP patterns (ribotypes) of all *Enterococcus hermanniensis* sp. nov. strains examined and of *Enterococcus* type strains generated by *Eco*RI (a) and *Hind*III (b). Numerical analyses of the patterns are presented as dendrograms; left-hand side of the *Eco*RI and *Hind*III banding patterns possesses high molecular masses, < 23 kbp, and right-hand side, > 1000 bp.

were run in a Global IR<sup>2</sup> 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 BIONUMERICS 3.0 software package (Applied Maths). In the BLAST analyses, isolates LMG 12317<sup>T</sup>, LMG 13617 and 334 showed the highest 16S rRNA gene sequence similarities to 16S rRNA gene sequences of strains classified as *Enterococcus pallens* (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. pallens* 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 NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 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 12317<sup>T</sup> and 334 was 37.1 and 36.6 mol%, respectively. The level of DNA-DNA relatedness between isolates 334 and LMG 12317<sup>T</sup> was 93.5%, whereas the levels between isolate LMG 12317<sup>T</sup> and *E. pallens* 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 12317<sup>T</sup> 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% bromcresol 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 Devriese & 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 12317<sup>T</sup> 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



**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

Species: 1, *E. hermanniensis* sp. nov.; 2, *E. avium*; 3, *E. pseudoavium*; 4, *E. raffinosus*; 5, *E. malodoratus*; 6, *E. gilvus*; 7, *E. pallens*. 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).

Characteristic	1	2	3	4	5	6	7
Growth at:							
10 °C	+	D	+	D	+	+	+
45 °C	-	+	+	+	D	+	+
Group D antigen	-	D	-	D	+	+	+
Acid production from:							
L-Arabinose	-	+	-	+	-	-	+
Dulcitol	-	D	-	-	D	ND	ND
Glycerol	-	+	-	+	D	+	+
Inositol	-	-	-	+	-	ND	ND
Lactose	-	+	+	+	+	+	+
Melibiose	-	-	-	+	+	+	+
Raffinose	-	-	-	+	+	+	+
Sorbitol	-	+	+	+	+	+	+
L-Sorbose	-	+	+	+	+	+	+

tonsils. Several *Enterococcus* species, together with some unidentified *E. avium*-like isolates, have been isolated from canine tonsils (Devriese *et al.*, 1992). Most enterococcal species are known to be associated with the intestines and their presence elsewhere is usually regarded as an indicator of faecal contamination. *E. faecalis*, *E. faecium*, *Enterococcus durans* and *Enterococcus hirae* have previously been associated with the gut microbiom of poultry (Devriese *et al.*, 1991). It is also possible that *E. hermanniensis* occurs in the gastrointestinal tract of broiler chickens and dogs, which has resulted in the contamination of poultry products and its occurrence in canine tonsils.

### 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 α-haemolysis. Strains grow slowly on azide-containing enterococcal selective media as typical, maroon colonies and cause blackening of bile-aesculin 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 α-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-xylose, glycogen 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-xyloside, D-raffinose, sucrose, sorbitol, L-sorbose, tagatose, D-turanose, xylitol or D-xylose 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 12317<sup>T</sup> (=CCUG 48100<sup>T</sup>), 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<sup>TM</sup>/LMG).

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