Description of *Enterococcus canis* sp. nov. from dogs and reclassification of *Enterococcus porcinus* Teixeira *et al.* 2001 as a junior synonym of *Enterococcus villorum* Vancanneyt *et al.* 2001

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Strains from anal swabs and chronic otitis externa in dogs were shown to be phylogenetically related to the *Enterococcus faecium* species group. They shared a number of phenotypic characteristics with these species, but they could be easily differentiated by biochemical reactions. In addition, the canine strains were unusual in their nearly complete failure to grow on sodium azide-containing enterococci-selective media and in their Voges–Proskauer reactions (usually negative). By using 16S rRNA sequencing and DNA–DNA hybridization of representative strains, as well as tDNA interspace gene PCR and SDS-PAGE of whole-cell proteins, the group of canine strains was shown to constitute a novel enterococcal species. The name *Enterococcus canis* sp. nov. is proposed for this species, with LMG 12316^T (= CCUG 46666^T) as the type strain. Concurrently, the taxonomic situation and nomenclatural position of *Enterococcus porcinus* were investigated. As no phenotypic or genotypic differences were found between this species and *Enterococcus villorum*, the name *E. porcinus* is considered to be a junior synonym of *E. villorum*.

INTRODUCTION

As the number of species described in the genus *Enterococcus* has increased, traditional phenotypic identification of this genus using genus-specific characteristics has become exceedingly difficult, if not impossible (Devriese *et al.*, 1993, 2002). Many strains do not possess the Lancefield group D antigen, several species do not grow on commonly used enterococci-selective media or at 10 or 45 °C, and characteristics such as growth in 6.5% NaCl and on aesculin bile agar or production of pyrrolidonyl arylamidase have become less useful as distinguishing characteristics. Two other easy reactions have been proposed to this end: ribose acidification and acetoin production (Voges–Proskauer; VP) (Devriese & Pot, 1995). However, these tests have also proved not to be universally applicable. In the present study, a group of mostly VP-negative,

enterococcal-like strains isolated from dogs was shown to belong to a novel species of the genus *Enterococcus*. In addition, synonymy of *Enterococcus porcinus* with *Enterococcus villorum* was demonstrated.

METHODS

Strains. Two strains, LMG 12315 and LMG 12316^T, were isolated in mixed culture from two cases of chronic otitis externa in dogs. The remaining 11 strains, LMG 21545, LMG 21546, LMG 21547, LMG 21548, LMG 21549, LMG 21550, LMG 21551, LMG 21552, LMG 21553, LMG 21554 and LMG 21555, were isolated from anal swabs of individually kept healthy dogs that all belonged to different owners. Strains were isolated on Columbia CNA blood agar (Oxoid) and selected on the basis of their identical or nearly identical tDNA-PCR fingerprints, which were different from those of all lactic acid bacteria available in our database (Baele *et al.*, 2000, 2001, 2002).

Phenotypic analysis. Growth tests were carried out as described by Švec *et al.* (2001). Acidification of carbohydrates was recorded after 3 days incubation in API 50 CH galleries (bioMérieux) under paraffin cover. Lancefield antigens were detected by using the Streptococcal Grouping kit (Oxoid). Biochemical reactions were determined by using the BBL Crystal Gram-positive ID kit (Becton Dickinson) and the API 20 STREP system (bioMérieux). VP tests were duplicated using Voges–Proskauer diagnostic tablets (Rosco).

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Abbreviations: tDNA-PCR, tRNA intergenic length polymorphism analysis; VP, Voges–Proskauer.

The GenBank/EMBL/DDBJ accession numbers for the 16S DNA sequences reported in this paper are X76177 and AY156090.

SDS-PAGE of whole-cell proteins. After incubation of cells for 24 h on MRS agar medium (Oxoid), whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis were performed by using the GelCompar software package, versions 3.1 and 4.0, respectively (Applied Maths).

tRNA intergenic length polymorphism analysis (tDNA-PCR). Genomic DNA was extracted by suspending a bacterial colony in 20 µl lysis buffer (0.25% SDS, 0.05 M NaOH). After heating at 95 °C for 5 min, 180 µl sterile distilled water was added and the lysate was centrifuged at 13 000 g for 5 min. The spacers between the tRNA genes were amplified using primers T3B (5'-AGGTC-GCGGGTTTCAATCC-3') and T5A (5'-AGTCCGGTGCTTAACCACTGAG-3'), which are directed against the conserved edges of the tRNA genes. PCR mixtures and cycle conditions were the same as previously described (Baele *et al.*, 2000). One primer (T3B) was fluorescently labelled to enable detection during capillary electrophoresis of the DNA fragments, which was done with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Electrophoretograms obtained with GENESCAN software (Applied Biosystems) were compared to our database by using in-house software (Baele *et al.*, 2000).

16S rRNA gene sequence analysis. To amplify the 16S rRNA gene, PCR was performed by using the conserved primers $\alpha\beta$ -NOT (5'-TCAAAGTAGGACCGAGTC-3') and ω_{MB} (5'-TACCTTGTTACTTACCCCCA-3') and the Taq PCR Master Mix kit (Qiagen). A QiaQuick PCR Purification kit (Qiagen) was used to purify the PCR product. Subsequent sequencing reactions were performed by using the BigDye Terminator Sequencing kit (Applied Biosystems) and the primers *Gamma, *O, PD and 3, as described by Coenye *et al.* (1999); the sequences were determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Phylogenetic analysis was done by using the program GENEBASE (Applied Maths). Pairwise alignment homologies were calculated and a dendrogram was constructed using the neighbour-joining method.

DNA base composition. Cells were cultivated in MRS broth (Oxoid) for 24 h at 37 °C. DNA was extracted from 0.5–0.75 g cells (wet weight), using the protocol described by Marmur (1961) with the following modifications: (i) cells suspended in Tris/HCl buffer were treated with lysozyme overnight (8 mg ml⁻¹) before addition of SDS; (ii) lysed cells were treated with proteinase K (360 mg l⁻¹; Merck) for 2 h at 37 °C. For determination of DNA base composition, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by HPLC, using a Waters SymmetryShield C₈ column maintained at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

DNA–DNA hybridization. High-molecular-weight native DNA was prepared as described for the determination of DNA base composition. DNA–DNA hybridizations were performed by using a modification of the microplate method described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS 7000 Bio Assay Reader (PerkinElmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA that was non-covalently bound to the microplate wells. Hybridizations were performed under stringent conditions at 34 °C in hybridization solution (2 × SSC, 5 × Denhardt's solution, 2.5% dextran sulphate, 50% formamide, 100 µg denaturated salmon sperm DNA ml⁻¹, 1250 ng biotinylated probe DNA ml⁻¹).

RESULTS AND DISCUSSION

tDNA-PCR

Typical tDNA spacer fragment lengths were 62.5, 65.4, 66.3, 85.8, 86.8, 155.9, 252.6, 257.5, 258.8 and 341.7 bp. These fragments served to distinguish the unidentified dog strains from other known enterococcal species included in the database of the Department of Bacteriology, Faculty of Veterinary Medicine, University of Ghent, Belgium, established by Baele *et al.* (2000).

16S rRNA gene sequences. The 16S rDNA sequences of strains LMG 12316^T and LMG 21553, isolated respectively from chronic otitis externa and an anal swab from dogs, demonstrated a similarity of 99.6%. Highest inter-species 16S rDNA homologies were shown with the type strains of the *Enterococcus faecium* species group (98.4–99.0%). This close phylogenetic similarity is also reflected by their phenotypic resemblance to the unidentified dog strains (Table 1). Somewhat lower similarities were shown between the canine strains and members of the *Enterococcus avium* species group (97.9–98.3%).

Table 1. Phenotypic characteristics that differentiate *E. canis* and other species of the *E. faecium* group from the remaining phylogenetic *Enterococcus* species groups

Species groups: 1, *E. faecium*, including *E. canis*, *E. durans*, *E. hirae*, *E. villorum* and *E. mundtii*; 2, *E. faecalis*, including *Enterococcus haemoperoxidus*, *Enterococcus moraviensis* and *Enterococcus ratti*; 3, *Enterococcus cecorum*, including *Enterococcus columbae*; 4, *E. avium*, including *Enterococcus pseudoavium*, *Enterococcus raffinosus*, *Enterococcus malodoratus* and *Enterococcus gilvus*; 5, *Enterococcus dispar*, including *Enterococcus asini* and *Enterococcus pallens*; 6, *Enterococcus gallinarum*, including *Enterococcus casseliflavus*. Data for taxa other than *E. canis* were obtained from Devriese & Pot (1995), de Vaux *et al.* (1998), Švec *et al.* (2001), Teixeira *et al.* (2001), Vancanneyt *et al.* (2001) and Tyrrell *et al.* (2002). Characteristics are scored as: +, positive; D, strain-dependent; D+, usually positive; D–, usually negative; –, negative.

Characteristic	1	2	3	4	5	6
Motility	–	–	–	–	–	+
Pyrrolidonyl arylamidase	+	+	–	+	D	+
Alkaline phosphatase	–	–	D+	–	–†	–
Acid from:						
Adonitol	–	–*	–	D†	–‡	–
D-Arabitol	–	–*	D	–†	–‡	–
Inulin	–	–	+	–	–	+
Melezitose	–	D+*	D	D†	–‡	D–
Melibiose	D	–§	+	D	D	+
L-Sorbose	–	–	–	+	D	–

*Not reported for *E. ratti*.

†Not reported for *E. gilvus*.

‡Not reported for *E. pallens*.

§Variable in *E. ratti*.

The sequence of *E. villorum* strain LMG 12287^T was found to be identical to that of *E. porcinus* strain ATCC 700913^T (Fig. 1). The description of *E. villorum* (Vancanneyt *et al.*, 2001) was based on the study of this strain (among others). As the name *E. villorum* was published earlier than the name *E. porcinus*, the latter name is to be considered to be a junior synonym of *E. villorum*.

DNA base compositions

The DNA G+C contents of strains LMG 12316^T, LMG 12315 and LMG 21553 were found to be 41.7, 43.0 and 42.3 mol%, respectively. These values are higher than those reported for other members of the *E. faecium* group, which range from 35 to 40 mol% (Collins *et al.*, 1984, 1986; Schleifer & Kilpper-Bälz, 1984; Farrow & Collins, 1985; Vancanneyt *et al.*, 2001).

DNA–DNA hybridization experiments

The level of DNA–DNA binding between the canine strains LMG 12316^T and LMG 12315 was 86 %. Despite the fact that 16S rDNA sequence homologies between the unidentified dog strains and reference enterococcal species were relatively high, very low DNA–DNA reassociation levels

were observed. Homology levels of 7–13 % between the dog strains and members of the *E. faecium* species group (*E. faecium* LMG 11423^T, *Enterococcus durans* LMG 10746^T, *Enterococcus hirae* LMG 6399^T, *Enterococcus mundtii* LMG 10748^T and *E. villorum* LMG 12287^T) clearly demonstrate that the isolates from dogs represent a separate genospecies and are distinct from other recognized members of the *E. faecium* group.

SDS-PAGE of whole-cell proteins

Duplicate protein extracts were prepared, to check the reproducibility of the growth conditions and the extract preparation. The level of correlation between duplicate protein patterns was $r \geq 0.95$. The whole-cell protein profiles of the dog strains were initially compared with patterns of >800 enterococcal strains, representing all currently described *Enterococcus* species; the unidentified isolates formed a separate cluster (Pot & Janssens, 1993; data not shown). A dendrogram obtained after average linkage cluster analysis, showing the separateness of the dog isolates from members of the *E. faecium* species group, is shown in Fig. 2. The dog strains formed a single and separate cluster ($r \geq 0.91$) and displayed almost-identical electrophoretic patterns. The pattern of the *E. porcinus* strain was highly similar to that of the *E. villorum* strain.

Biochemical activity

The closest phylogenetic relatives of the unknown dog isolates, *E. faecium*, *E. durans*, *E. hirae*, *E. mundtii* and *E. villorum*, which constitute the *E. faecium* species group (Williams *et al.*, 1991), have a number of common characteristics that are useful for phenotypic identification. These differential characteristics can also be used to differentiate the dog strains from other groups of enterococcal species (Table 1). However, the dog strains are unusual in that they do not grow on commonly used enterococcal selective media that contain 0.04 % sodium azide (NaN₃) and in that most strains give a negative VP reaction. These characteristics can be used along with other tests, notably certain carbohydrate acidifications, to differentiate the unidentified dog strains from related enterococcal species (Table 2). *E. porcinus*, described by Teixeira *et al.* (2001), is not included in this table because its biochemical activity is identical to that of *E. villorum*.

It is evident from the results of polyphasic taxonomic study that the unidentified enterococcal-like isolates from dogs represent a hitherto unknown species of the genus *Enterococcus*. Phylogenetically, the novel species is a member of the *E. faecium* group and displays relatively high 16S rRNA gene sequence similarities (approx. 98.4–99 %) with other species within this group. The values of 1–1.6 % sequence divergence between the dog bacterium and members of the *E. faecium* group are somewhat low. However, many other genomically distinct enterococcal species that display comparable or even less 16S rRNA gene sequence divergence have been described (e.g. Williams

Fig. 1. Distance matrix tree showing the phylogenetic relationships of *Enterococcus* species based on 16S rDNA sequence comparisons. *Enterococcus solitarius* was used as the outgroup and bootstrap probability values (%) are indicated at the branch-points (500 tree replications).

Fig. 2. Protein profiles of *Enterococcus* strains and corresponding dendrogram, derived from the unweighted pair group average linkage of correlation coefficients, r (expressed as a percentage value for convenience).

Table 2. Characteristics that differentiate *E. canis* from other species of the *E. faecium* group

Species: 1, *E. canis*; 2, *E. faecium*; 3, *E. hirae*; 4, *E. durans*; 5, *E. villorum*; 6, *E. mundtii*. Based on data from Devriese & Pot (1995), Vancanneyt *et al.* (2001), Devriese *et al.* (2002) and the present study. Characteristics are scored as: +, positive; D, strain-dependent; D+, usually positive; D-, usually negative; -, negative.

Characteristic	1	2	3	4	5	6
Yellow pigment	-	-	-	-	-	+
VP reaction	D-	+	+	+	+	+
Resistance to 0.04% NaN ₃	-	+	+	+	+	+
Arginine hydrolysis (API)	-	+	+	+	+	+
Acid from:						
L-Arabinose	+	+	-	-	-	+
Gluconate	+	D	-	-	-	-
Mannitol	+	D+	-	-	-	+
Melibiose	-	D+	D+	D+	D+	+
Methyl- α -D-glucoside	+	-	-	-	-	-
Raffinose	-	-*	D-	D-	-	D+
Sucrose	D-	D+	+	D	-	+
D-Xylose	D+	D-	-	-	D+	+

*Strains from chickens are usually positive for production of acid from raffinose.

et al., 1991; Švec *et al.*, 2001). DNA-DNA pairing studies showed unequivocally that the canine isolates represent a different genomic species from other members of the *E. faecium* group. Further support for the distinctiveness of the novel species comes from phenotypic evidence; in particular, protein profiling confirms the separateness of the dog isolates from all currently defined enterococcal species. The novel species can also be readily distinguished from other enterococci by using a combination of physiological and biochemical tests. Therefore, based on both phylogenetic and phenotypic evidence, we propose that the isolates from dogs are assigned to *Enterococcus canis* sp. nov.

To date, *E. canis* has only been isolated from dogs. Although this species may occur in chronic and complicated otitis externa cases in this host, it probably does not play a pathogenic role. This type of lesion is often contaminated by faecal flora.

Description of *Enterococcus canis* sp. nov.

Enterococcus canis (ca'nis. L. gen. n. *canis* of a dog).

Cells are non-motile, facultatively anaerobic, Gram-positive, slightly elongated cocci that occur in pairs, short chains or small groups. Colonies on blood agar are circular, smooth

and surrounded by narrow, sharply demarcated zones of α -haemolysis. Growth is optimal at 37 °C, slower at 42, 30 and 25 °C and unaffected by the absence or presence of 5 % CO₂. Strains grow in 6.5 % NaCl broth. On Edward's *Streptococcus* selective medium, brownish aesculin-degrading colonies are formed. No growth is seen after 1 day on Slanetz–Bartley medium, but after 2 days, pinpoint-sized colonies are formed without any evidence of tetrazolium-chloride reduction. Abundant growth and blackening are observed in aesculin bile agar. Starch is not digested. Positive results are obtained in API tests for leucine arylamidase and pyrrolidonyl arylamidase (hydrolysis of L-pyrroglutamic acid-AMC in Crystal, with some weak reactions) and in the following Crystal reactions: hydrolysis of L-valine-AMC, L-phenylalanine-AMC, 4MU- α -D-glucoside, L-tryptophan-AMC, *p*-nitrophenyl- β -D-glucoside, *p*-nitrophenyl phosphate and *p*-nitrophenyl- β -D-cellobioside. Acid is produced in the API 20 STREP and/or API 50 CH kits in tests with glycerol (often delayed), L-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, β -gentiobiose, gluconate and 2-ketogluconate (often weak). Most strains react positively in tests with α -methyl-D-glucoside (10/13 strains), D-arabinose (11/13 strains) and D-xylose (8/13 strains). Few strains are positive for acid production from sucrose (2/13 positive), trehalose (1/13 positive) and starch (4/13 strains with weak reactions). Strain- or method-dependent reactions are observed with arginine (negative in API 20 STREP and Rosco; 6/13 strains positive in Crystal galleries) and VP (negative in API 20 STREP, 2/13 strains positive with Rosco tablets). Strains are negative in API tests for hippurate, β -glucuronidase, β -galactosidase and alkaline phosphatase, and in Crystal tests for hydrolysis of L-valine-AMC, 4MU-phosphate, 4MU- β -D-glucuronide, L-isoleucine-AMC and urease. Acid is produced from erythritol, L-xylose, adonitol, L-sorbose, rhamnose, dulcitol, sorbitol, α -methyl-D-mannoside, melibiose, inulin, melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D- and L-fucose, D-tagatose, D- and L-arabitol and 5-ketogluconate. The DNA G + C content is 41.7–43.0 mol%.

The type strain, LMG 12316^T (=CCUG 46666^T), was isolated from anal swabs and chronic otitis externa in dogs.

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