Enterococcus alcedinis sp. nov., isolated from common kingfisher (Alcedo atthis)

Petra Frolková,1 Pavel Švec,2 Ivo Sedláček,2 Ivana Mašlaňová,2 Jitka Černohlávková,2 Anuradha Ghosh,3 Ludek Zurek,3 Tomáš Radiměřský1 and Ivan Literák1,4

Correspondence
Petra Frolková
p.frolkova@seznam.cz

1Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, 612 42 Brno, Czech Republic
2Czech Collection of Microorganisms, Department of Experimental Biology, Faculty of Science, Masaryk University, Tvrdeho 14, 602 00 Brno, Czech Republic
3Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA
4CEITEC VFU, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, 612 42 Brno, Czech Republic

Two Gram-positive, catalase-negative bacterial strains were isolated from the cloaca of common kingfishers (Alcedo atthis). Repetitive sequence-based PCR fingerprinting using the (GTG)5 primer grouped these isolates into a single cluster separated from all known enterococcal species. The two strains revealed identical 16S rRNA gene sequences placing them within the genus Enterococcus with Enterococcus aquimarinus LMG 16607T as the closest relative (97.14 % similarity). Further taxonomic investigation using sequencing of the genes for the superoxide dismutase (sodA), phenylalanyl-tRNA synthase alpha subunit (pheS) and the RNA polymerase alpha subunit (rpoA) as well as application of whole-cell protein fingerprinting, automated ribotyping and extensive phenotyping confirmed that both strains belong to the same species. Based on data from this polyphasic study, these strains represent a novel species of the genus Enterococcus, for which the name Enterococcus alcedinis sp. nov. is proposed. The type strain is L34T (=CCM 8433T=LMG 27164T).

Enterococci are Gram-positive, facultatively anaerobic, catalase-negative cocci in the family Enterococcaceae (Švec & Devriese, 2009). They colonize a wide range of different environments. Enterococci are natural members of the intestinal microbiota of humans and other mammals. Representatives of this genus have also been reported in birds, reptiles and insects (Aarestrup et al., 2002; Shoemaker et al., 2006). Moreover, they are widespread in water and on plants. Enterococci are used in manufacturing food products such as starter cultures and probiotics; however, they are also known to cause food spoilage (Aarestrup, 2006) and, in the recent past, enterococci have become important nosocomial pathogens harbouring resistance to multiple antibiotics (van den Bogaard et al., 2002).

In this study, two enterococcal strains were isolated in the frame of a project dealing with the microbial community of free-living birds. Strains L17 and L34T originated from the cloaca of two common kingfisher (Alcedo atthis, Alcedinidae) nestlings from the localities Hrádek (49° 44’ N 14° 55’ E) (L17) and Káčov (49° 42’ N 14° 53’ E) (L34T), Vlašim, Czech Republic, in June 2009. Cloacal smears were aseptically sampled with sterile cotton swabs, placed into Amies transport medium (Oxoid) and Anaerobe medium (Oxoid), and transported to the laboratory. Samples were cultivated on blood agar (Oxoid) with colistin (10 mg l−1) and nalidixic acid (10 mg l−1) at 37 °C for 48 h. Individual colonies were selected, purified and stored at −70 °C. The investigated strains were deposited in the Czech Collection of...
Microorganisms (CCM) (Masaryk University, Brno, Czech Republic, www.sci.muni.cz/ccm) and in the BCCM/LMG Bacteria Collection (Gent University, Belgium, http://bccm.belspo.be/index.php) as L17 (=CCM 8434=LMG 27165) and L34T (=CCM 8433T=LMG 27164T).

Genotypic screening of isolates was performed using repetitive-element PCR (rep-PCR) fingerprinting with the (GTG)$_5$ primer, which was evaluated as a suitable tool for the identification of Enterococcus spp. (Svec et al., 2005). Total genomic DNA isolated by alkaline extraction was amplified by PCR using the (GTG)$_5$ primer (5’-GTGGTGTTGGTTGGT-3’) as described previously (Svec et al., 2008). The resulting fingerprints were processed by the BioNumerics v. 6.6 software (Applied-Maths), and compared with the in-house (GTG)$_5$-PCR database of the CCM containing multiple type and reference strains covering all recognized species of the genus Enterococcus. The two isolates revealed identical fingerprints and were grouped in a single cluster separated from all reference strains included in the database. Fig. S1 (available in IJSEM Online) shows the (GTG)$_5$ fingerprints obtained from the isolates and demonstrates their separation from other members of the genus Enterococcus.

The 16S rRNA gene was amplified using universal primers EU16SrRNA/F (5’-AGAGTTTGATCITGGCTCAG-3’) and EU16SrRNA/R (5’-ACGGITACCTTGTTACGACTT-3’) (Cook & Meyers, 2003). The initial denaturation was for 1 min at 94 °C, followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 54 °C for 30 s and elongation at 72 °C for 1 min. The final extension step was for 8 min at 72 °C. A single DNA band with length of ~ 1500 bp was observed in both strains on 1.5% agarose gel with ethidium bromide. The PCR product was purified with the Jet Quick gel extraction spin kit (Genomed) and Gel/PCR DNA fragments extraction kit (Geneaid). Quantification of DNA was performed using a nanodrop ASP-3700 machine (ACTGene), and both strands were sequenced by Macrogen. The novel sequences and reference sequences obtained from the GenBank database were aligned using BioEdit (Hall, 1999) followed by reconstruction of a phylogenetic tree using the neighbour-joining method inferred from 1000 replicates (Saitou & Nei, 1987) with the help of MEGA5 software (Tamura et al., 2011). Strains L17 and L34T shared 100% 16S rRNA gene sequence similarity. Enterococcus aquimarinus LMG 16607T was the closest relative with 97.14% similarity. Fig. 1 shows the phylogenetic relationships between strains L17 and L34T and the type strains of selected enterococcal species representing different phylogenetic lineages. A more complete tree containing all recognized enterococcal species is shown in Fig. S2.

Sequencing of the superoxide dismutase (sodA) gene was performed according to Frolková et al. (2012). The internal fragment of the sodA gene was amplified using degenerate

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains L17 and L34T within the genus Enterococcus. Tetragenococcus solitarius 885-78T was used as an outgroup. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 1% sequence divergence.](image-url)
strains L17 and L34T within the genus *Enterococcus* and purification as well as processing of the obtained 2005). Chromosomal DNA isolation, PCR amplification for the species delineation of enterococci (Naser subunit (phaS) and RNA polymerase alpha subunit (rpoA) genes has been shown to be a valuable tool for the species delineation of enterococci (Naser et al., 2005). Chromosomal DNA isolation, PCR amplification and purification as well as processing of the obtained sequences and reconstruction of the dendrograms using MEGA5 were performed as described by Švec et al. (2012). The novel sequences and reference sequences from the GenBank database were aligned and the phylogenetic tree was reconstructed as described above. Strains L17 and L34T shared 100% sodA gene sequence similarity and were separated from the remaining species of the genus *Enterococcus*. The closest relative was *E. aquimarinus* CCM 7283T, which shared 82.34% sodA gene sequence similarity with the studied strains. The resulting tree based on phylogenetic analysis of the sodA gene is shown in Fig. 2(a) and a more complete tree including all known *Enterococcus* spp. is shown in Fig. S3.

Sequence analysis based on the partial phenylalanyl-tRNA synthase alpha subunit (phaS) and RNA polymerase alpha subunit (rpoA) genes was performed as described above. The results revealed high levels of phaS (98.73%) and rpoA (100%) gene sequence similarity between strains L17 and L34T and differentiated them from the remaining species of the genus *Enterococcus*. Fig. 2(b, c) shows the positions of strains L17 and L34T within the genus *Enterococcus* resulting from analysis of the phaS and rpoA genes, respectively. Figs S4 and S5 show more complete phylogenetic trees based on phaS and rpoA gene sequences, respectively, including all known species of the genus *Enterococcus*.

Whole-cell protein fingerprinting proved to correlate well with DNA–DNA hybridization experiments (Vandamme et al., 1996). This method was applied to confirm that strains L17 and L34T are members of the same species and to show that they are different from their phylogenetically closest relative, *E. aquimarinus*. Cells cultivated on brain heart infusion (BHI) agar (Oxoid) for 24 h at 37 °C were harvested and disrupted using a Labsonic M ultrasonic homogenizer (Sartorius) and the protein extracts were separated using SDS-PAGE according to the protocol described by Pot et al. (1994). Numerical analysis of the protein profiles obtained was performed using BioNumerics software v.6.6. The resulting dendrogram was reconstructed using Pearson’s similarity coefficient with the UPGMA method. Strains L17 and L34T revealed similar profiles (84% similarity), confirming that they are members of the same species. The two novel strains were differentiated at a similarity level of 18% from their phylogenetically closest relative, *E. aquimarinus*, represented by strains CCM 7283T and CCM 7284 (Fig. 3). Automated ribotyping with the EcoRI restriction enzyme was performed using the RiboPrinter microbial characterization system (DuPont Qualicon) in accordance with the standard protocol provided by the manufacturer. The obtained ribopatterns were imported into the BioNumerics v. 6.6 software using a Load samples import script obtained from the manufacturer. The dendrogram was reconstructed based on calculation of Pearson’s correlation coefficients with the UPGMA. Strains L17 and L34T revealed almost identical profiles (98% similarity) and were separated from the remaining enterococci. Fig. S6 shows ribotype patterns obtained from analysed strains and demonstrates their differentiation from the type strains of other species of the genus *Enterococcus*.

Genotyping of isolates L17 and L34T was performed by pulsed-field gel electrophoresis (PFGE) performed according to Ghosh et al. (2012) with several modifications. DNA was digested with 20 U *Sma*I for 4 h at 37 °C. PFGE was performed with an initial pulse time of 1 s and final time of 20 s at 200 V for 17.5 h. Comparison of the PFGE fingerprints was carried out using BioNumerics v. 6.6 software and the results are shown in Fig. S7. *Enterococcus faecium* ATCC 19434T was used as the reference. The new isolates formed two different pulsotypes which revealed that they are representatives of different strains.

Phenotypic studies of strains L17 and L34T were performed using conventional tests and commercial identification kits. Catalase production was tested using an ID Colour Catalase kit (bioMérieux). Motility was studied on a stab-panel (Biolog) according to the manufacturers’ instructions. Differentiation of the investigated strains from *E. aquimarinus* could be achieved based on a few phenotypic tests. Strains L17 and L34T were positive for acid production from glucose and gluconate was tested according to Šperber & Swan (1976). Extensive phenotypic characterization was performed using commercial identification kits API 20 Strep, API 50 CH and API ZYM (bioMérieux), STREPTOtest 24 (Erba Lachema), and Biolog Identification System using GP2 MicroPlate Gram-positive identification test panel (Biolog) according to the manufacturers’ instructions. Differentiation of the investigated strains from *E. aquimarinus* could be achieved based on a few phenotypic tests. Strains L17 and L34T were positive for acetoin production and acidification of 5-ketogluconate and ribose but negative for growth in 6.5% NaCl, production of x-galactosidase, and acid production from l-arabinose, melibiose and raffinose. *E. aquimarinus* revealed opposite results for these tests. Detailed results for the phenotypic characterization of the investigated isolates are given in the species description below.

Determination of the DNA G+C content was performed by HPLC. DNA was isolated using a modification of the method of Marmur (1961) and hydrolysed with P1 nuclease, and nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The
resulting deoxyribonucleotides were analysed by HPLC (Shimadzu) as described by Tamaoka & Komagata (1984). Chromatograms were analysed using the CLARITY (version 2.4.1.93) software package (DataApex). The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) to thymidine (dT) according to the method of

Fig. 2. Neighbour-joining phylogenetic trees based on sodA (a), pheS (b) and rpoA (c) gene sequences showing the positions of strains L17 and L34T within the genus Enterococcus. Tetragenococcus solitarius CIP 103330T and LMG 12890T were used as outgroups. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bars, 5% sequence divergence.
Described by Mesbah et al. (1989). The DNA G+C content of strain L34<sup>T</sup> was 35.1 mol%.

Data from the present polyphasic taxonomic study indicate that strains L17 and L34<sup>T</sup> represent a novel species of the genus Enterococcus, for which the name Enterococcus alcedinis sp. nov. is proposed.

**Description of Enterococcus alcedinis sp. nov.**

*Enterococcus alcedinis* [al.ce’di.nis. N.L. n. *Alcedo* -inis *Alcedo*, a scientific zoological generic name; N.L. gen. n. *alcedinis* of *Alcedo*, isolated from *Alcedo atthis* (common kingfisher)].

The description of the species is based on two strains. Cells are Gram-reaction-positive, spherical or ovoid, non-spore-forming, non-motile cocci, occurring predominantly in pairs, short chains or irregular clusters. Colonies on BHI agar supplemented by 7% sheep blood are non-pigmented, circular with whole margins, convex, smooth, glistening, and reach 1–2 mm in diameter at 37°C after 24 h cultivation. Weak growth of pinpoint colonies with positive aesculin reaction occurs on kanamycin aesculin azide agar at 37°C after 24 h. No growth is observed on Slanetz-Bartley agar. Facultatively anaerobic, growing at 15 and 42°C, but not at 10 or 45°C or in the presence of 6.5% NaCl. Gas production from glucose and gluconate is negative. Does not react with the Lancefield group D antigen antisera. Positive for aesculin hydrolysis, Voges-Proskauer test (acetoin), pyrrolidinyl arylamidase and leucine arylamidase production. Negative for hippurate hydrolysis, catalase, urease and arginine dihydrolase production. Positive for chymotrypsin, esterase (C4), esterase lipase (C8), lipase (C14), naphthol-AS-BI-phosphophydrase and β-galactosidase, but negative for valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Acid is produced from ribose, D-xylene, galactose, glucose, fructose, mannose, mannotol, N-acetylglucosamine, arbutin, salicin, cellobiose, maltose, sucrose, trehalose and 5-keto-gluconate, but not from glycerol, erythritol, D-arabinose, L-arabinose, L-xylene, adonitol, methyl α-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, melibiose, raffinose, starch, xylitol, β-gentiobiose, D-lyxose, D-fructose, L-fucose, D-arabitol, L-arabinol, pullulan, glucanate or 2-keto-glucanate. Shows the following variable biochemical reactions.

- Strain L34<sup>T</sup> is positive for acid production from melezitose, and delayed positive reactions (5 days) are noted for D-tagatose and D-turanose acidification while strain L17 is negative for these three tests. Shows variable results for β-glucosidase (positive in STREPTO Test 24 and negative in API ZYM), phosphatase (positive in API ZYM and API 20 Strep but negative in STREPTO Test 24), acid production from inulin (positive in STREPTO Test 24 and API 20 Strep but negative in API 50 CH kit), acid production from glycerogen (positive in API 20 Strep but negative in API 50 CH kit) and acid production from lactose (positive in API 50 CH kit but negative in STREPTO Test 24 and API 20 Strep).
- Utilizes dextrin, N-acetyl-D-glucosamine, arbutin, D-fructose, D-galactose, α-D-glucose, D-mannitol, D-mannose, D-psicose, salicin, sucrose, trehalose, inosine, thymidine and uridine (Biolog Identification System with GP2 MicroPlate test panel), but not α-cyclodextrin, β-cyclodextrin, glycerogen, inulin, mannans, Tween 40, Tween 80, N-acetyl-β-D-mannosamine, amygdalin, L-arabinose, D-arabitol, L-fucose, D-galacturonic acid, gentiobiose, D-gluconic acid, myo-inositol, α-lactose, lactulose, maltotriose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, D-rhamnose, sedoheptulose, D-sorbitol, stachyose, D-tagatose, turanose, xylitol, D-xylene, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylactic acid, α-ketoglutaric acid, β-ketoglutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinic acid, 3-cinnamic acid, succinic acid, N-acetyl-L-glutamic acid, and D-phenylacetic acid.
L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycol L-glutamic acid, L- pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2'-deoxyadenosine, adenosine-5'- monophosphate, thymidine-5'-monophosphate, uridine- 5'-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate or D-L-α-glycerol phosphate.

The type strain is L34T (=CCM 8433T=LMG 27164T), isolated from the cloaca of the common kingfisher (Alcedo attis). The DNA G+C content of strain L34T is 35.1 mol%. L17, isolated from a similar source, is a second strain of the species.

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References


