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### **How to cite this manuscript**

If you make reference to this version of the manuscript, use the following information:

Frolkova, P., Svec, P., Sedlacek, I., Maslanova, I., Cernošlavkova, J., Ghosh, A., Zurek, L., Radimersky, T., & Literak, I. (2013). *Enterococcus alcedinis* sp nova, isolated from common kingfisher (*Alcedo atthis*). Retrieved from <http://krex.ksu.edu>

### **Published Version Information**

**Citation:** Frolkova, P., Svec, P., Sedlacek, I., Maslanova, I., Cernošlavkova, J., Ghosh, A., Zurek, L., Radimersky, T., & Literak, I. (2013). *Enterococcus alcedinis* sp nova, isolated from common kingfisher (*Alcedo atthis*). *International Journal of Systematic and Evolutionary Microbiology*, 63, 3069-3074.

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**Digital Object Identifier (DOI):**10.1099/ijs.0.049833-0

**Publisher's Link:** [http://ijs.sgmjournals.org/content/63/Pt\\_8/3069.abstract](http://ijs.sgmjournals.org/content/63/Pt_8/3069.abstract)

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

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**Running title:** *Enterococcus alcedinis* sp. nov.

**Abbreviations:** *sodA*, manganese-dependent superoxide dismutase gene; rep-PCR, repetitive-  
element polymerase chain reaction; *pheS*, phenylalanyl-tRNA synthase alpha subunit gene;  
*rpoA*, RNA polymerase alpha subunit gene

**Contents Category:** New Taxa; Firmicutes and related organisms

The GenBank database accession numbers for the 16S rRNA gene sequences of *Enterococcus alcedinis* CCM 8434 and CCM 8433<sup>T</sup> are JX948101 and JX948102. The GenBank database accession numbers for the *sodA* gene sequences are JX948095 and JX948096. The GenBank database accession numbers for the *pheS* and *rpoA* gene sequences obtained in this study are JX948097, JX948098 and JX948099, JX948100 respectively.

Seven supplementary figures are available with the online version of this paper.

**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)<sub>5</sub> primer grouped these isolates into a single cluster separated from all known enterococcal species. Both strains revealed identical 16S rRNA gene sequences placing them within the genus *Enterococcus* with *Enterococcus aquimarinus* 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 the polyphasic data, these strains represent a novel species of the genus *Enterococcus*, for which the name *Enterococcus alcedinis* sp. nov. is proposed. The type strain is L34<sup>T</sup> (= CCM 8433<sup>T</sup> = LMG 27164<sup>T</sup>).**

Enterococci are Gram-positive, facultative 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 recent past, enterococci have become important nosocomial pathogens harboring resistance to multiple antibiotics (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. The strains L17 and L34<sup>T</sup> originated from the cloaca of two common kingfisher (*Alcedo atthis*, *Alcedinidae*) nestlings from the localities Hrádek (49°44'N, 14°55'E) (L17) and Kácov (49°42'N, 14°53'E) (L34<sup>T</sup>), Vlašim, Czech Republic in June, 2009. Cloacal smears were aseptically sampled with sterile cotton swab, placed into Amies transport medium (Oxoid, Hampshire, United Kingdom) and Anaerobe medium (Oxoid), and transported into the laboratory. Samples were cultivated on blood agar (Oxoid) with colistin (10 mg.l<sup>-1</sup>) and nalidixic acid (10 mg.l<sup>-1</sup>) at 37°C for 48 h. Individual colonies were selected, purified, and stored at -70°C. Investigated strains were deposited in the Czech Collection of Microorganisms (CCM) (Masaryk University, Brno, Czech Republic, [www.sci.muni.cz/ccm](http://www.sci.muni.cz/ccm)) and in the BCCM/LMG Bacteria Collection (Gent University, Belgium, <http://bccm.belspo.be>) as L17 (= CCM 8434 = LMG 27165) and L34<sup>T</sup> (= CCM 8433<sup>T</sup> = LMG 27164<sup>T</sup>).

Genotypic screening of isolates was performed using rep-PCR fingerprinting with the (GTG)<sub>5</sub> primer, which was evaluated as a suitable tool for the identification of *Enterococcus* spp. (Švec *et al.* 2005). The total genomic DNA isolated by alkaline extraction procedure was amplified by PCR using the (GTG)<sub>5</sub> primer (5'-GTG GTG GTG GTG GTG-3') as described previously (Švec *et al.*, 2008). The resulting fingerprints were processed by the BioNumerics

v. 6.6 software (Applied-Maths, Sint-Martens-Latem, Belgium), and compared with the in-house (GTG)<sub>5</sub>-PCR database of the Czech Collection of Microorganisms containing multiple type and reference strains covering all hitherto described *Enterococcus* spp. Both isolates revealed identical fingerprints and were grouped in a single cluster separated from all reference strains included in the database. Fig. S1 shows the (GTG)<sub>5</sub> 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 & Mayers, 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 1min. The final extension step was for 8 min at 72 °C. A single DNA band with the length of ~ 1500 bp was observed in both strains on 1.5% agarose gel with ethidium bromide. PCR product was purified with the Jet Quick gel extraction spin kit (Genomed, Löhne, Germany) and Gel/PCR DNA fragments extraction kit (Geneaid, New Taipei City, Taiwan). Quantification of DNA was performed by nanodrop ASP-3700 (ACTGene, Piscataway ,USA), and both strands were sequenced by Macrogen Comp, South Korea. Our sequences and reference sequences obtained from GenBank database were aligned using Bioedit (Hall, 1999) followed by construction of a phylogenetic tree using the neighbour-joining method inferred from 1000 replicates (Saitou and Nei, 1987) with the help of MEGA5 software (Tamura *et al.*, 2011). Strains L17 and L34<sup>T</sup> revealed 100% 16S rRNA gene sequence similarity to each other. *Enterococcus aquimarinus* was the closest relative with 97.14% similarity. Fig. 1 shows the phylogenetic relationships of strains L17 and L34<sup>T</sup> and selected enterococcal species representing different phylogenetic lineages. An elaborate tree containing all recognised enterococcal species is shown in Fig S2.

Sequencing of the *sodA* gene was performed according to Frolková *et al.* (2012). The internal fragment of the *sodA* gene was amplified using degenerate primers d1 (5'-CCI TAY ICI TAY GAY GCI YTI GAR CC-3') and d2 (5'-ARR TAR TAI GCR TGY TCC CAI ACR TC-3') (Poyart *et al.*, 2000). Purification of PCR product, quantification of DNA, and sequencing was performed as described above. Our sequences and reference sequences from the GenBank database database were aligned and the phylogenetic tree was constructed as described above. Strains L17 and L34<sup>T</sup> revealed 100% *sodA* sequence similarity and were separated from the remaining species of the genus *Enterococcus*. The closest species was *E. aquimarinus* revealing 82.34% similarity with the studied strains. The resulting tree based on phylogenetic analysis of the *sodA* gene is shown in Fig. 2a and the extended tree including all known *Enterococcus* spp. is shown in Fig. S3.

Sequence analysis based on the partial phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) genes has been shown as 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 construction of the dendrograms using the MEGA5 were performed as described by Švec *et al.* (2012). Our sequences and reference sequences obtained from the GenBank database database were aligned and the phylogenetic tree was constructed as described above. Results showed high similarity of *pheS* (98.73%) and *rpoA* (100%) genes between strains L17 and L34<sup>T</sup> and differentiated them from the remaining *Enterococcus* spp. Figs. 2b and 2c demonstrate position of our isolates within the genus *Enterococcus* resulting from the analysis of *pheS* and *rpoA* genes, respectively. Figs. S4 and S5 show extended phylogenetic trees for *pheS* and *rpoA*, respectively, including all known *Enterococcus* spp.

Whole-cell protein fingerprinting proved to correlate well with DNA-DNA hybridization experiments (Vandamme *et al.*, 1996). This method was applied to confirm that

the investigated strains L17 and L34<sup>T</sup> are members of the same species and to show that they are different from the phylogenetically closest relative *E. aquimarinus*. The cells cultivated on Brain Heart Infusion (BHI) agar (Oxoid) for 24 h at 37°C were harvested and disrupted by Labsonic M ultrasonic homogenizer (Sartorius) and the protein extracts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the protocol described by Pot *et al.* (1994). Numerical analysis of obtained protein profiles was performed using BioNumerics software v.6.6. The resulting dendrogram was constructed using the Pearson's correlations similarity coefficient with the unweighted pair group method using arithmetic averages (UPGMA). Strains L17 and L34<sup>T</sup> revealed similar profiles (84% similarity) confirming that they are members of the same species. Similarity level of 18% differentiated both strains from the phylogenetically closest species *E. aquimarinus* represented by strains CCM 7283<sup>T</sup> 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. Obtained ribopatterns were imported into the BioNumerics v. 6.6 software using a Load samples import script obtained from the manufacturer. The dendrogram was constructed based on calculation of Pearson's correlation coefficients with the UPGMA. Strains L17 and L34<sup>T</sup> 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 *Enterococcus* spp.

Genotyping of isolates L17 and L34<sup>T</sup> was performed by pulsed-field gel electrophoresis (PFGE) performed according to Ghosh *et al.* (2012) with several modifications. DNA was digested with 20 U of *SmaI* for 4 h at 37°C. The PFGE was performed with initial pulse time for 1 s and final time for 20 s at 200 V for 17.5 h.

Comparison of the PFGE fingerprints was carried out by BioNumerics v. 6.6 software and are shown in Fig. S7. *E. faecium* ATCC 19434<sup>T</sup> was used as the reference. Our isolates formed two different pulsotypes which revealed that they are representatives of different strains.

Phenotypic studies on strains L17 and L34<sup>T</sup> were performed using conventional tests and commercial identification kits. Catalase production was tested using an ID Colour Catalase kit (bioMérieux, Craponne, France). Motility was studied on a stab-inoculated semi-solid medium as described by Švec *et al.* (2012). Growth at different temperatures and with 6.5% NaCl was examined in BHI broth (Oxoid) for up to 5 days. Lancefield D antigen was determined using a Strep kit (DiaMondiaL, Dublin, Ireland). Production of gas from glucose and gluconate was tested according to Sperber & 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, Brno, Czech Republic), and Biolog Identification System using GP2 MicroPlate Gram-positive identification test panel (Biolog) according to the manufacturers' instructions. Differentiation of investigated strains from the phylogenetically related *E. aquimarinus* species may be achieved using a few phenotypic tests. Strains L17 and L34<sup>T</sup> were positive for acetoin production and acidification of 5-ketogluconate and ribose but negative for growth in 6.5% NaCl, production of  $\alpha$ -galactosidase and acid production from L-arabinose, melibiose and D-raffinose. *Enterococcus aquimarinus* revealed opposite results for these tests. Detailed results for the phenotypic characterization of the investigated isolates are mentioned in the species description below.

Determination of GC content of DNA was performed by HPLC. DNA was isolated by modification of the Marmur method (Marmur, 1961), hydrolysed with P1 nuclease and nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleotides were analysed by HPLC System (Shimadzu) following condition as described earlier (Tamaoka & Komagata, 1984). Chromatograms were analysed



using the CLARITY (Version 2.4.1.93) software package (DataApex Ltd.). GC content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah *et al.* (1989). The DNA G+C content of strain L34<sup>T</sup> was 35.1 mol%.

The presented polyphasic taxonomic study revealed 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-positive spherical or ovoid cocci, occurring predominantly in pairs and in short chains or in irregular clusters, non-sporeforming and non-motile. 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 esculin reaction was obtained on kanamycin aesculin azide agar at 37°C after 24 h. No growth was observed on Slanetz-Bartley agar. The species is a facultative anaerobe and grows 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. It does not react with the Lancefield group D antigen antisera. Esculin hydrolysis, Voges-Proskauer test (acetoin), pyrrolidonyl arylamidase, and leucin arylamidase production positive. Hippurate hydrolysis, catalase, urease, and arginin dihydrolase production negative. Chymotrypsin, esterase (C 4), esterase lipase (C 8), lipase (C 14), naphthol-AS-Bi-phosphohydrolase and  $\beta$ -galactosidase positive. Valine arylamidase, cystine arylamidase, trypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase, N-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase negative. Acid

is produced from ribose, D-xylose, galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine, arbutin, salicin, cellobiose, maltose, sucrose, trehalose and 5-keto-gluconate. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol,  $\alpha$ -methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol,  $\alpha$ -methyl-D-mannoside,  $\alpha$ -methyl-D-glucoside, amygdalin, melibiose, raffinose, starch, xylitol,  $\beta$ -gentiobiose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, pullulan, gluconate, and 2-keto-gluconate. The variable biochemical reactions revealed by *E. alcedinis* are as follows. The type strain L34<sup>T</sup> was positive for acid production from melezitose and delayed positive reactions (5 days) were noted for D-tagatose and D-turanose acidification while strains L17 was negative for these three tests. Discrepant results were obtained for  $\beta$ -glucosidase (positive in STREPTOtest 24 and negative in API ZYM), phosphatase (positive in API ZYM and API 20 Strep while negative in STREPTOtest 24), acid from inulin (positive in STREPTOtest 24 and API 20 Strep and negative in API 50 CH kit), acid from glycogen (positive in API 20 Strep and negative in API 50 CH kit) and acid from lactose (positive in API 50 CH and negative in STREPTOtest 24 and API 20 Strep). The Biolog Identification System with the GP2 MicroPlate test panel revealed the ability to utilize dextrin, N-acetyl-D-glucosamine, arbutin, D-fructose, D-galactose,  $\alpha$ -D-glucose, D-mannitol, D-mannose, D-psicose, salicin, sucrose, D-trehalose, inosine, thymidine, and uridine. Negative utilization tests results were recorded for  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin, glycogen, inulin, mannan, Tween 40, Tween 80, N-acetyl- $\beta$ -D-mannosamine, amygdalin, L-arabinose, D-arabitol, L-fucose, D-galacturonic acid, gentiobiose, D-gluconic acid, m-inositol,  $\alpha$ -D-lactose, lactulose, maltotriose, D-melezitose, D-melibiose,  $\alpha$ -methyl-D-galactoside,  $\beta$ -methyl-D-galactoside, 3-methyl glucose,  $\alpha$ -methyl-D-glucoside,  $\beta$ -methyl-D-glucoside,  $\alpha$ -methyl-D-mannoside, D-raffinose, L-rhamnose, sedoheptulosan, D-sorbitol, stachyose, D-tagatose, turanose, xylitol, D-xylose, acetic acid,  $\alpha$ -

hydroxybutyric acid,  $\beta$ -hydroxybutyric acid,  $\gamma$ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid,  $\alpha$ -ketoglutaric acid,  $\beta$ -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, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2'-deoxy adenosine, adenosine-5'-monophosphate, thymidine-5'-monophosphate, uridine-5'-monophosphate, D-fructose-6-phosphate,  $\alpha$ -D-glucose-1-phosphate, D-glucose-6-phosphate and D-L- $\alpha$ -glycerol phosphate. The DNA G+C content of strain L34<sup>T</sup> is 35.1 mol%.

Type strain L34<sup>T</sup> (= CCM 8433<sup>T</sup> = LMG 27164<sup>T</sup>) was isolated from the cloaca of the common kingfisher (*Alcedo attis*).

## Acknowledgements

This study was supported by the project CEB (CZ.1.07/2.3.00/20.0183) from the Ministry of Education, Youth and Sports of the Czech Republic and the project “CEITEC - Central European Institute of Technology” (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund. We thank Pavel Čech for sampling kingfishers.

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