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Enterococcus aquimarinus sp. nov., isolated from sea water

Pavel Švec,[1] Marc Vancanneyt,[2] Luc A. Devriese,[3] Sabri M. Naser,[2,4] Cindy Snauwaert,[2] Karen Lefebvre,[2] Bart Hoste[2] and Jean Swings[2,4]

Correspondence
Pavel Švec
mpavel@sci.muni.cz

[1]Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Tvrdého 14, 602 00 Brno, Czech Republic

[2,4]BCCM/LMG Bacteria Collection[2] and Laboratory of Microbiology, Faculty of Sciences[4], Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium

[3]Laboratory of Veterinary Bacteriology and Mycology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

Two enterococcal strains LMG 16607^T and LMG 16612 originating from sea water were analysed in a polyphasic taxonomic study. Both strains, assigned as *Enterococcus* sp. in the BCCM/LMG culture collection, possessed analogous protein profiles, but these were different from all other enterococcal species. 16S rRNA gene sequence analysis of one strain showed the highest similarity, 96·9–96·1 %, with its closest phylogenetic neighbours *Enterococcus saccharolyticus*, *Enterococcus sulfureus*, *Enterococcus saccharominimus* and *Enterococcus italicus*. Further genomic analysis by $(GTG)_5$-PCR fingerprinting and sequence analysis of the housekeeping gene phenylalanyl-tRNA synthase (*pheS*) and distinct biochemical features confirmed that the two strains represent a novel enterococcal species for which the name *Enterococcus aquimarinus* sp. nov. is proposed. The type strain is LMG 16607^T (=CCM 7283^T).

Enterococci are Gram-positive cocci inhabiting various environments. They are generally considered to be commensal inhabitants of warm-blooded animals, including humans, but are also isolated from reptiles and insects. Moreover, they occur on different kinds of food and can be found on plants and in water (Devriese *et al.*, 1992). It has become impossible to achieve a reliable identification using classical biochemical tests as the number of enterococcal species with validly published names has increased. For some of the more recently described *Enterococcus* species, characteristics traditionally considered to be typical for the genus, e.g. acetoin production, ribose acidification, resistance to 6·5 % NaCl and growth at 10 and 45 °C, are no longer applicable (Devriese *et al.*, 1993; Devriese & Pot, 1995; Domig *et al.*, 2003). Other methods, such as SDS-PAGE analysis of whole-cell proteins, have been used intensively as routine and validated identification systems for enterococci (Devriese *et al.*, 2002; De Graef *et al.*, 2003; Vancanneyt *et al.*, 2004).

The present study deals with two strains presumptively assigned as *Enterococcus* sp. in the large BCCM/LMG in-house database containing SDS-PAGE whole-cell protein profiles of all described lactic acid bacteria. Both strains, LMG 16607^T (=CCM 7283^T) and LMG 16612 (=CCM 7284), originate from sea water and were deposited in BCCM/LMG in 1995 via bioMérieux with strain numbers API 8407116 and API 8407104, respectively. BioMérieux collected the strains in 1984 from the Istituto Superiore di Sanita of Roma, Italy. Further inquiries to determine the origin of the strains were unsuccessful. The taxonomic position of both sea-water isolates was further elucidated in this work. The reference strains used for comparison in this study were obtained from the BCCM/LMG bacteria collection (http://www.belspo.be/bccm/).

To determine the phylogenetic position of the sea-water isolates, 16S rRNA gene sequence analysis was performed on one strain, LMG 16607^T, as described by Vancanneyt *et al.* (2004). The sequence obtained (a continuous stretch of 1510 bp) was aligned with reference sequences obtained from GenBank and edited by using the BioEdit program (Hall, 1999) and ForCon (Raes & Van De Peer, 1999). Evolutionary distances were calculated using the Jukes & Cantor evolutionary model and a phylogenetic tree was

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Abbreviation: rep-PCR, repetitive-element polymerase chain reaction.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Enterococcus aquimarinus* sp. nov. LMG 16607^T is AJ877015.

A phylogenetic tree based on *pheS* gene sequence comparisons of LMG 16607^T and LMG 16612 with other enterococcal species is available as supplementary material in IJSEM Online.

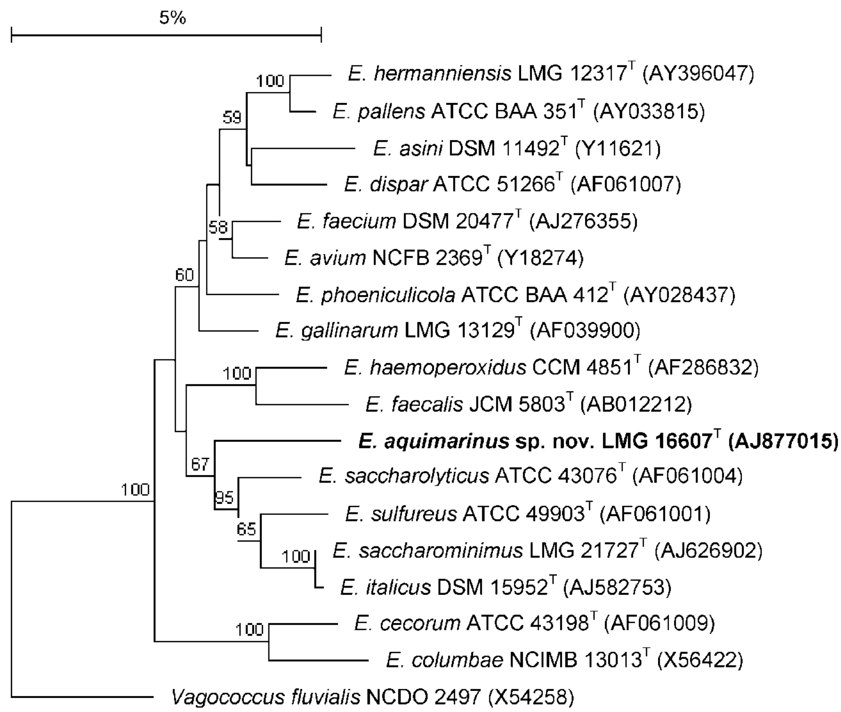

Fig. 1. Distance matrix tree based on 16S rRNA gene sequence comparisons showing the phylogenetic relationships of *E. aquimarinus* sp. nov. and selected enterococcal species representing different phylogenetic lineages. The *Vagococcus fluvialis* (X54258) sequence was used as the outgroup. Bootstrap percentage values (500 tree replications) higher than 50 % are indicated at the branch points. GenBank accession numbers in parentheses. Bar, 5 % sequence divergence.

constructed using the neighbour-joining method with TREECON software (Van De Peer & De Wachter, 1994). Based on 16S rRNA gene sequencing, it was found that strain LMG 16607^T represented a separate lineage distantly related to the other enterococci (see Fig. 1). The closest similarities were obtained with *Enterococcus saccharolyticus* (96·9 %), *Enterococcus sulfureus* (96·3 %), *Enterococcus saccharominimus* (96·5 %) and *Enterococcus italicus* (96·1 %). The 16S rRNA gene similarity of strain LMG 16607^T with its closest neighbour, *E. saccharolyticus*, was slightly below 97 %, a value that is generally accepted as the boundary value for the delineation of species (Stackebrandt & Goebel, 1994). This indicates that both strains represent a separate species and excludes the need for DNA–DNA hybridizations. The separate species status of the sea-water isolates was confirmed by other validated phenotypic and genotypic methods as detailed below.

A comparison of whole-cell protein profiles from the sea-water isolates with all described enterococcal species grouped them separately. Whole-cell protein extracts were prepared from cells grown for 24 h on MRS agar (Oxoid) at 37 °C. SDS-PAGE, densitometric analysis of protein profiles and numerical analysis were performed in accordance with the procedure described by Pot *et al.* (1994). Fig. 2 shows protein profiles obtained from strains LMG 16607^T and LMG 16612 and demonstrates their separate position from the phylogenetically closest species *E. saccharolyticus*, *E. sulfureus*, *E. saccharominimus* and *E. italicus*.

The separate taxonomic position of the sea-water isolates was also confirmed by using repetitive-element PCR (rep-PCR), a genomic screening method, using the $(GTG)_5$ primer. DNA preparation, amplification, separation, analysis of the rep-PCR fingerprint profiles by BioNumerics version 4.0 software and dendrogram construction were performed as described by Gevers *et al.* (2001). Strains LMG 16607^T and LMG 16612 showed visually identical $(GTG)_5$-PCR patterns and they were clearly separated from fingerprints obtained from other enterococcal species (Fig. 3).

Naser *et al.* (2005) investigated the usefulness of housekeeping genes as alternative phylogenetic and identification tools for all enterococcal species based on recent *in silico* studies of the whole genome sequences of different bacterial groups. Primers for the gene that encodes phenylalanyl-tRNA synthase, *pheS*, were designed using 12 *pheS* gene sequences from lactic acid bacteria. The *pheS* primers

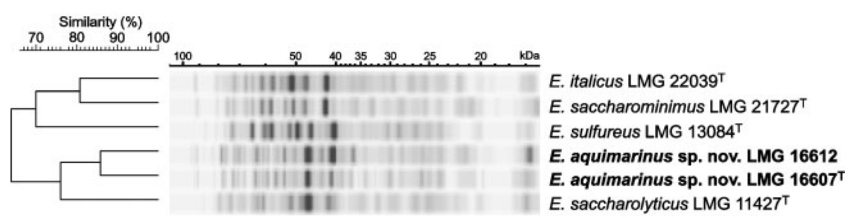

Fig. 2. Protein profiles of strains LMG 16607^T, LMG 16612 and the type strains of phylogenetically related species. The dendrogram was constructed by UPGMA linkage of correlation coefficients (*r*, expressed for convenience as percentage values).

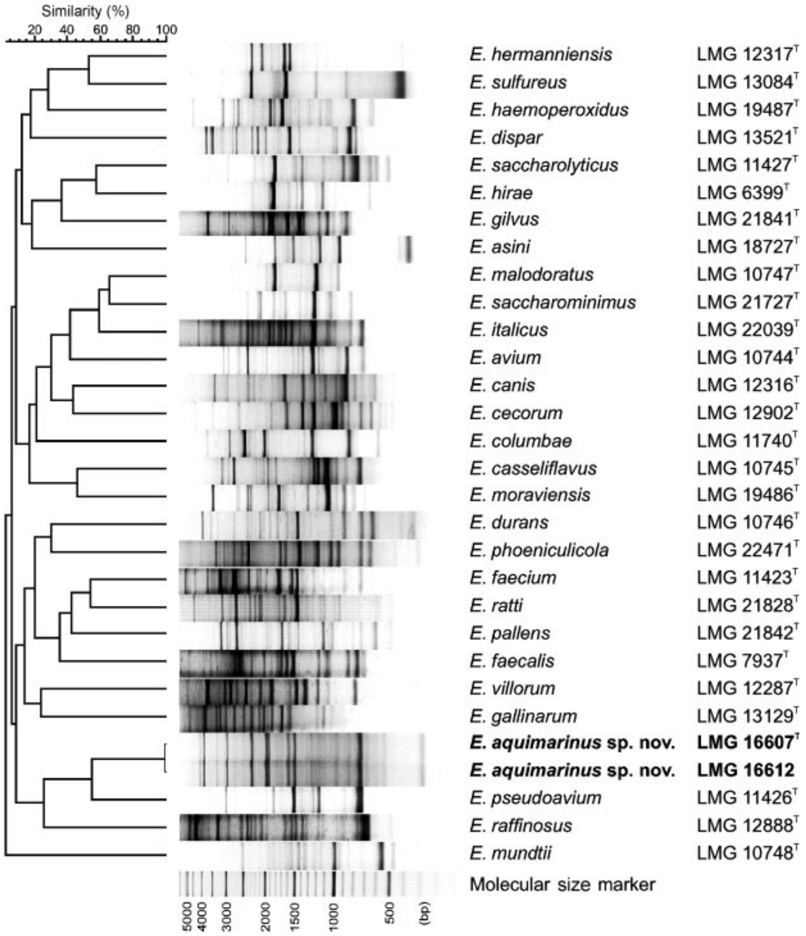

Fig. 3. Dendrogram based on $(GTG)_5$-PCR results obtained from strains LMG 16607^T and LMG 16612 and from the type strains representing all enterococcal species with validly published names. The dendrogram was constructed with the Pearson correlation coefficient using the UPGMA method (*r*, expressed for convenience as percentage similarity values).

enabled the amplification and sequencing of a 455 bp *pheS* fragment from all of the described *Enterococcus* species (Table 1). The conditions for amplification and sequencing reactions are described elsewhere (Naser *et al.*, 2005). Consensus sequences were determined using two reads for the *pheS* gene. Different enterococcal species had a maximum of 86 % *pheS* gene sequence similarity, indicating that *pheS* is a fast-evolving clock and a valuable tool for the identification of all the currently described enterococcal species. Evaluation of intraspecies variation showed that *pheS* genes had a high degree of homogeneity, at least 97 %, within strains of the same species. Sequence analysis of the *pheS* gene confirmed that the sea-water isolates LMG 16607^T and LMG 16612 are members of a single species (100 % similarity) and were clearly differentiated from all other enterococcal species with validly published names (see Supplementary Fig. S1 in IJSEM Online). The closest species was *E. sulfureus*, with 79·4 % sequence similarity. Our results show that the comparison of *pheS* gene sequence data is an efficient screening method for the detection of novel enterococcal species.

Table 1. Primers that enabled the amplification and sequencing of a 455 bp *pheS* fragment from all *Enterococcus* species with validly published names

Primer	Sequence (5′–3′)	Position
pheS-21-F	CAYCCNGCHCGYGAYATGC	557
pheS-22-R	CCWARVCCRAARGCAAARCC	1031
pheS-23-R	GGRTGRACCATVCCNGCHCC	968

For DNA base composition analysis, high-molecular-mass DNA from bacterial cells grown on Todd–Hewitt broth (Oxoid) at 37 °C was isolated as described by Mannerová *et al.* (2003). Degradation of the isolated DNA into nucleosides and their separation by HPLC was performed as described by Vancanneyt *et al.* (2004). The DNA G + C content of strains LMG 16607^T and LMG 16612 was 38·7 mol%.

Growth and biochemical tests were carried out as described by Švec *et al.* (2001). A search for Lancefield antigens was performed with a Streptococcal grouping kit (Oxoid). The results are given in the species description below. The two analysed strains revealed identical biochemical profiles, enabling them to be differentiated from other enterococcal species. Strains LMG 16607^T and LMG 16612 could be differentiated from the majority of known enterococcal species by their inability to produce acid from ribose. Ribose

Table 2. Phenotypical tests useful for the differentiation of *E. aquimarinus* sp. nov. from the closest phylogenetic relatives, *E. sulfureus*, *E. italicus*, *E. saccharominimus* and *E. saccharolyticus*, and from another ribose-negative species, *E. asini*

Taxa: 1, *E. aquimarinus* sp. nov.; 2, *E. saccharolyticus*; 3, *E. sulfureus*; 4, *E. italicus*; 5, *E. saccharominimus*; 6, *E. asini*. Data for *E. aquimarinus* sp. nov. were obtained in this study; data for other taxa were reported by de Vaux *et al.* (1998), Farrow *et al.* (1984), Fortina *et al.* (2004), Martinez-Murcia & Collins (1991) and Vancanneyt *et al.* (2004). +, Positive; −, negative.

Characteristic	1	2	3	4	5	6
Pigment production	−	−	+	−	−	−
Pyrrolidonyl arylamidase	+	−	+	+	+	+
β-Glucuronidase	−	+	−	−	−	−
Hippurate hydrolysis	−	−	−	−	−	+
Acidification of:						
D-Arabitol	−	+	−	−	−	−
L-Arabinose	+	−	−	−	−	−
Gluconate	−	−	+	−	−	−
Inulin	+	+	−	−	−	−
Melezitose	−	+	+	−	−	−
Melibiose	+	+	+	−	−	−
D-Raffinose	+	+	+	−	−	−
Ribose	−	+	+	−	−	−
Sucrose	+	+	+	+	+	−
D-Xylose	+	−	−	−	−	+

acidification is considered to be typical for the genus *Enterococcus*, although several of the more recently described species, e.g. *E. asini*, *E. italicus* and *E. saccharominimus*, are negative for this trait. Table 2 shows phenotypical tests that are useful for the differentiation of strains LMG 16607^{T} and LMG 16612 from their closest phylogenetic relatives, *E. sulfureus*, *E. italicus*, *E. saccharominimus* and *E. saccharolyticus*, and from another ribose-negative species, *E. asini*.

The overall results of the present study allowed us to assign strains LMG 16607^{T} and LMG 16612 to a novel species for which we propose the name *Enterococcus aquimarinus* sp. nov.

Description of *Enterococcus aquimarinus* sp. nov.

Enterococcus aquimarinus (a.qui.ma.ri′nus. L. fem. n. *aqua* water; L. adj. *marinus* of the sea; N.L. masc. adj. *aquimarinus* pertaining to sea water).

Cells are elongated, often lanceolate. They occur singly or in small groups and predominantly in pairs. The type strain grows equally well at 25 and 42 °C. Addition of 5 % CO_2 does not enhance growth. Colonies on Columbia agar with sheep blood are small (1–2 mm), not pigmented, translucent, shining and regular. Greening haemolysis. Turbid growth in liquid media with a deposit. Grows in the presence of 6·5 % NaCl. Grows and produces blackening on bile aesculin agar. Poor growth on Slanetz and Bartley medium containing 0·04 % sodium azide. Not motile. No detectable Lancefield antigens. Positive in tests for pyrrolidonyl arylamidase and for α- and β-galactosidase. Arginine hydrolysis is negative. Acid is produced from L-arabinose, D-xylose, galactose, glucose, fructose, mannose, *N*-acetylglucosamine, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, starch and turanose. Negative in Voges–Proskauer test and tests for hippurate, β-glucuronidase and alkaline phosphatase. No acid produced from glycerol, erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl β-glucoside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-glucoside, amygdalin, melezitose, glycogen, xylitol, β-gentiobiose, lyxose, tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate. DNA G+C content is 38·7 mol%.

The type strain, LMG 16607^{T} (=CCM 7283^{T}), was isolated from sea water.

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