Enterococcus silesiacus sp. nov. and *Enterococcus termitis* sp. nov.

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Three enterococci constituted two aberrant branches after numerical analysis of $(GTG)_5$-PCR fingerprints: analogous patterns were found for two water isolates, strains W213 and W442^T, and a separate position was found for an isolate from the gut of a termite, strain LMG 8895^T. 16S rRNA gene sequence analysis classified all three strains in the *Enterococcus faecalis* species group. Further sequencing analysis of the housekeeping gene *pheS* (encoding the phenylalanyl-tRNA synthase α-subunit) and whole-cell-protein analysis confirmed a distinct position for the two water isolates and the termite strain, respectively. DNA–DNA hybridization experiments and distinct phenotypic features between the strains studied and representatives of the *E. faecalis* species group confirmed novel species status, respectively, for the two water isolates, strains W213 and W442^T, and for strain LMG 8895^T. The names *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. are proposed for the novel taxa, with W442^T (=CCM 7319^T=LMG 23085^T) and LMG 8895^T (=CCM 7300^T) as the respective type strains.

Enterococci generally occur as inhabitants of the human and animal intestinal tract, but they are also common in fermented food and are isolated from the environment (Devriese & Pot, 1995). Although enterococci are considered beneficial and safe members of the population of various fermented products (Giraffa, 2002), they are involved in a variety of human nosocomial infections (Teixeira & Facklam, 2003). The genus is phylogenetically subdivided into a number of species groups. Within these species groups, enterococcal species share certain physiological and phenotypical characteristics that may be useful for their identification (Devriese *et al.*, 1993). Although this identification approach is still valuable for the most common species, for some of the more recently described species a combination of phenotypic and molecular methods is required for reliable identification (Domig *et al.*, 2003; Devriese *et al.*, 2002). In the present paper, we describe two novel enterococcal species by using a polyphasic approach.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Enterococcus termitis* LMG 8895^T, *E. silesiacus* W442^T and W213 are AM039968, AM039966 and AM039967, respectively.

A neighbour-joining tree based on the *pheS* gene sequences and protein profiles of strains W213, W442^T and LMG 8895^T are available as supplementary material in IJSEM Online.

Strains W213 (=CCM 7318=LMG 23084) and W442^T (=CCM 7319^T=LMG 23085^T) were isolated from drinking water in the region of Silesia in the Czech Republic during a routine microbiological water analysis performed by filtration of a 10 ml water sample through Millipore filters (max. pore size 0·45 μm) and cultivation of the filters on Slanetz–Bartley agar plates for 24 h at 37 °C as described by Švec & Sedláček (1999). Strain LMG 8895^T (=CCM 7300^T) was isolated from the gut of a termite and was originally described as *Lactococcus lactis* subsp. *lactis*. SDS-PAGE of proteins, however, already revealed (results not shown) that the strain was a member of the enterococci. All other type and reference strains included in this study were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/).

Genotypic characterization was performed using rep-PCR fingerprinting with the $(GTG)_5$ primer as described by Švec *et al.* (2005). $(GTG)_5$-PCR fingerprints obtained were normalized using BioNumerics (version 4.0) and compared with available profiles in an in-house database (BCCM/LMG Bacteria Collection) covering all described enterococcal species. Strains W213 and W442^T showed analogous patterns, and strain LMG 8895^T occupied a separate branch distinct from all other reference strains (Fig. 1).

Analysis of the complete 16S rRNA gene sequence of strains W213, W442^T and LMG 8895^T was performed as described

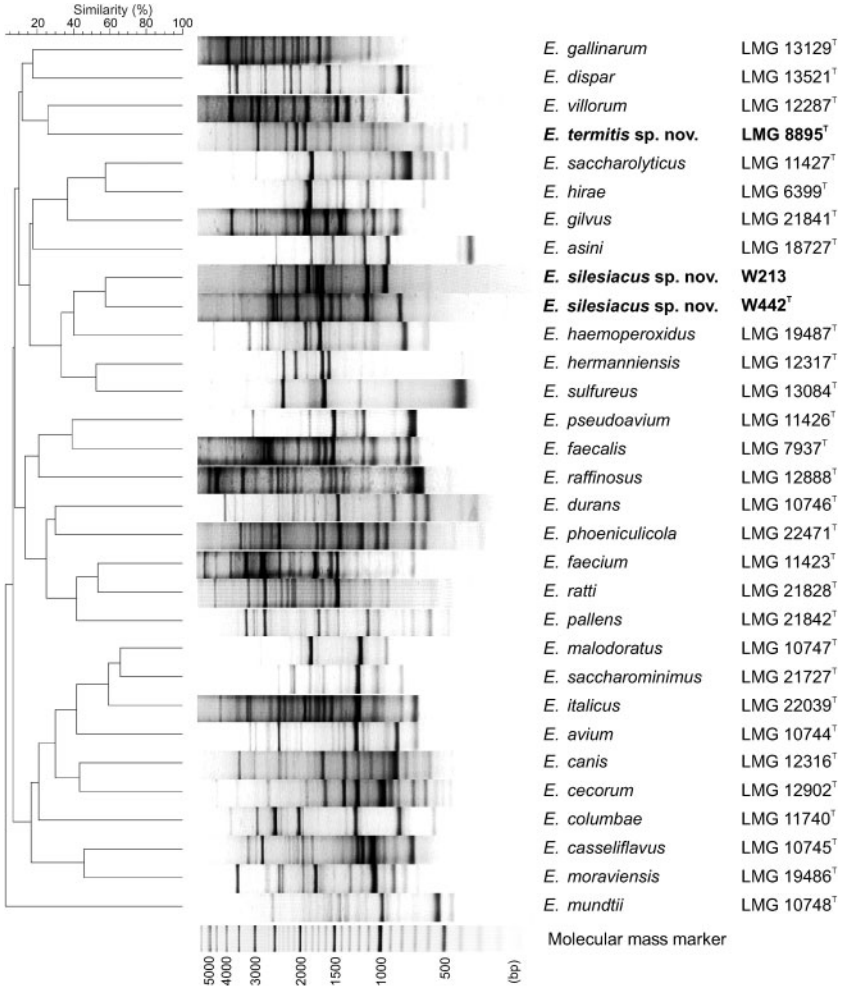

Fig. 1. $(GTG)_5$-PCR fingerprints obtained from strains W213, W442^T and LMG 8895^T and from the type strains representing all recognized enterococcal species. The dendrogram was calculated with Pearson's correlation coefficient using UPGMA clustering method (*r*, expressed for convenience as percentage similarity values).

by Vancanneyt *et al.* (2004). The sequences obtained and reference sequences (downloaded from the GenBank database) were aligned by using the BioEdit software (Hall, 1999). Evolutionary distances were calculated using the Jukes–Cantor evolutionary model (Jukes & Cantor, 1969) and a phylogenetic tree was constructed using the neighbour-joining method with the TREECON software (Van De Peer & De Wachter, 1994). The tree topology obtained with the neighbour-joining method was evaluated and confirmed by the maximum-parsimony analysis using BioNumerics (version 4.0). The phylogenetic analysis placed the three strains in the *Enterococcus faecalis* species group (Fig. 2), which accommodates *E. faecalis*, *Enterococcus haemoperoxidus* and *Enterococcus moraviensis* (Švec *et al.*, 2001). Strains W213 and W442^T showed 99·9 % 16S rRNA gene sequence similarity to each other and showed *E. haemoperoxidus* and *E. moraviensis* as their closest phylogenetic relatives with similarities ranging from 99·0 to 99·2 %. Similarly, strain LMG 8895^T showed 98·9 % 16S rRNA gene sequence similarity with *E. haemoperoxidus* and 98·8 % with *E. moraviensis* species. Sequence similarity between strain LMG 8895^T and strains W213 and W442^T was 99·3 %.

Amplification and partial sequencing of the *pheS* gene (encodes a phenylalanyl-tRNA synthase) were performed by using *pheS* primers: pheS-21-F (5′-CAYCCNGCHCGYGAYATGC-3′), pheS-22-R (5′-CCWARVCCRAARGCAAARCC-3′) and pheS-23-R (5′-GGRTGRACCATVCCNGCHCC-3′), which enabled the comparison of a 455 bp gene fragment. The *pheS* primers were designed based on a selection of the most conservative regions of the *pheS* gene sequence of representative lactic acid bacteria obtained from publicly available data of whole-genome-sequence projects. Sequencing primer designs, amplification conditions and sequencing parameters were performed as described by Naser *et al.* (2005). Although the sequences obtained represent only about half of the gene, Naser *et al.* (2005) demonstrated that this region shows sufficient diversity to distinguish individual species. Different enterococcal species have a maximum of 86 % *pheS* gene sequence similarity and the intraspecies variation showed a high degree of homogeneity of at least 97 % among strains of the same species. This suggested that *pheS* is a fast-evolving clock and a valuable tool for identification of enterococci; however, the topology obtained in the *pheS* dendrogram does not reflect

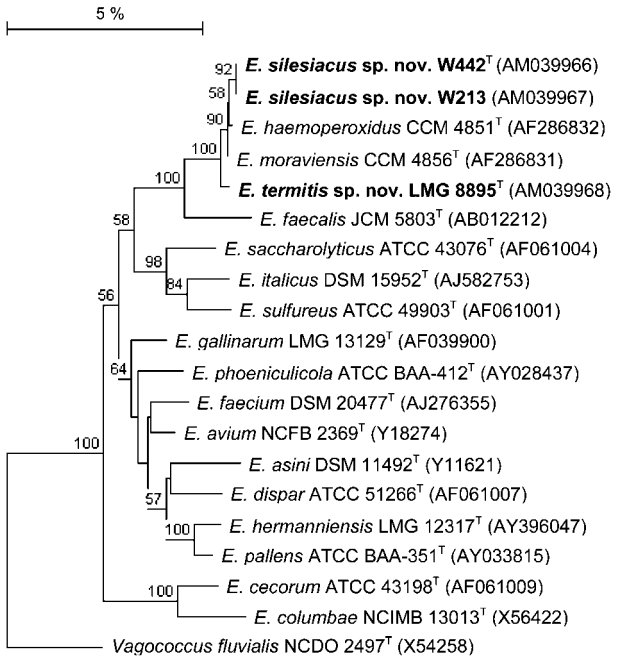

Fig. 2. Distance-matrix tree based on 16S RNA gene sequence comparisons showing the phylogenetic relationships of strains W213, W442^T and LMG 8895^T and selected enterococcal species representing phylogenetic neighbours and intraspecies lineages. The *Vagococcus fluvialis* (X54258) sequence was used as the outgroup. Bootstrap percentage values (500 tree replications) higher than 50 % are indicated at branch points. GenBank accession numbers are stated in parentheses. Bar, 5 % evolutionary distance.

the phylogenetic relationships revealed by 16S rRNA gene sequencing (Naser *et al.*, 2005). The *pheS* gene sequence analysis indicated that the two water isolates (W213 and W442^T) are members of a single species (99·3 % sequence similarity to each other). Comparison with reference strains revealed the highest sequence similarity of 86·8 % with *E. moraviensis* for both strains. Strain LMG 8895^T was differentiated from the above-mentioned isolates (sequence similarity of 84·0 %) and from all enterococcal type strains included in the database and constituted a single separate branch with the highest sequence similarity of 82·6 % with *E. moraviensis* as shown in Supplementary Fig. S1 (available in IJSEM Online). The latter data are an indication that both taxa might represent novel species.

Whole-cell-protein analysis was performed with cells grown on MRS agar (Oxoid) for 24 h at 37 °C. Protein extraction, electrophoresis, SDS-PAGE, densitometric analysis and further analysis of the profiles were performed following the procedure described by Pot *et al.* (1994). Protein profiles were compared to an in-house database (BCCM/LMG Bacteria Collection) comprising multiple representative strains of all described enterococcal species. The similarity between all pairs of traces was expressed by Pearson's product–moment correlation coefficient. UPGMA (unweighted pair group method using arithmetic averages) clustering was used for the construction of the dendrogram. Supplementary Fig. S2 (available in IJSEM Online) shows the whole-cell-protein profiles obtained from investigated strains as well as from their nearest phylogenetic neighbours *E. faecalis*, *E. haemoperoxidus* and *E. moraviensis*. The three new isolates were highly similar to *E. haemoperoxidus* and *E. moraviensis* reference strains. Still, the water isolates (strains W213 and W442^T) constituted a single cluster and showed minor differences from strain LMG 8895^T, *E. haemoperoxidus* and *E. moraviensis*.

The DNA base composition was determined for strains W213, W442^T and LMG 8895^T. Isolation of high-molecular-mass DNA from bacterial cells grown in Todd–Hewitt broth (Oxoid), degradation of the DNAs into nucleosides and their separation by HPLC were carried out as described by Vancanneyt *et al.* (2004). The DNA G+C content of strains W213, W442^T and LMG 8895^T were 35·6, 36·7 and 37·1 mol%, respectively. These results correspond to the DNA G+C content of the *E. faecalis* species group that range from 34·3 to 37·7 mol% (Švec *et al.*, 2001).

DNA–DNA hybridization experiments were performed between strains W213, W442^T and LMG 8895^T and *E. faecalis* LMG 7937^T, *E. moraviensis* LMG 19486^T and *E. haemoperoxidus* LMG 19487^T. High-molecular-mass DNA was isolated as described for determination of the DNA base composition and DNA–DNA hybridization experiments were performed in microdilution wells according to Vancanneyt *et al.* (2004). The hybridization temperature was 32 °C (calculated as described by Švec *et al.*, 2001). A high DNA-binding value of 93 % was found between strains W213 and W442^T and confirms that they represent a single species. DNA–DNA-binding levels between strains W213 and W442^T and *E. faecalis* LMG 7937^T, *E. moraviensis* LMG 19486^T and *E. haemoperoxidus* LMG 19487^T were 12 and 13 %, 41 and 43 % and 48 and 46 %, respectively. Binding levels between strain LMG 8895^T and *E. faecalis* LMG 7937^T, *E. moraviensis* LMG 19486^T and *E. haemoperoxidus* LMG 19487^T were 12, 26 and 30 %, respectively. The water isolates W213 and W442^T and strain LMG 8895^T showed binding levels between 25 and 26 %. These data confirm that the water isolates W213 and W442^T and strain LMG 8895^T represent two novel enterococcal species.

Growth and biochemical tests were carried out by using API 20 Strep and API 50CH commercial kits (bioMérieux) as well as by conventional tests described by Švec *et al.* (2001). Results are given in the species descriptions below. The species can be differentiated from their phylogenetically closest known relatives by using the tests listed in Table 1.

All results obtained in this study confirmed the analysed strains as members of two novel enterococcal species, for which the names *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. are proposed.

Table 1. Biochemical tests useful for differentiation of *Enterococcus silesiacus* sp. nov., *E. termitis* sp. nov. and their phylogenetic relatives assigned in the *E. faecalis* species group

Taxa: 1, *E. silesiacus* sp. nov.; 2, *E. termitis* sp. nov.; 3, *E. faecalis*; 4, *E. haemoperoxidus*; 5, *E. moraviensis*. Characteristics scored as: +, positive; −, negative; d, variable. Data described by Švec *et al.* (2001), de Vaux *et al.* (1998), Schleifer & Kilpper-Bälz (1984) and Devriese *et al.* (1983) or obtained in this study.

Characteristic	1	2	3	4	5
Arginine dihydrolase	+	−	+	+	−
Acetoin (VP test)	+	−	+	+	+
β-Galactosidase	+	−	−	−	d
Hippurate hydrolysis	−	−	+	+	+
Acid production from:					
Melezitose	−	−	d	+	+
L-Arabinose	d	−	−	−	+
Gluconate	d	+	d	−	−
Methyl α-D-glucopyranoside	−	+	−	+	+
Methyl α-D-mannopyranoside	−	+	−	−	−
Sucrose	−	−	+	+	+
D-Tagatose	−	−	+	−	+
D-Xylose	+	+	−	−	−

Description of *Enterococcus silesiacus* sp. nov.

Enterococcus silesiacus (si.le′si.a.cus. N.L. masc. adj. *silesiacus* pertaining to Silesia, the region in the Czech Republic from which the type strain originates).

Cells are Gram-positive, ovoid cocci, occurring in pairs, short chains or small groups. They elongate in the direction of the chains. Colonies on Columbia agar supplemented with sheep blood are non-pigmented, shiny, circular, smooth with entire margins and about 1 mm in diameter after 24 h of cultivation at 37 °C. The type strain of the species grows well on Todd–Hewitt agar and brain heart infusion (BHI) agar; growth on MRS medium is less abundant. Poor growth on Slanetz–Bartley medium containing 0·04 % sodium azide in small dark-red colonies. Growth with positive aesculin reaction on kanamycin/aesculin/azide agar and bile/aesculin agar. Non-motile. Growth occurs in BHI broth at 10 °C, no growth occurs at 45 °C and is weak in the presence of 6·5 % NaCl and at pH 9·6. Positive catalase reaction when cultivated on blood-containing agar, but catalase-negative on blood-free medium. Produces arginine dihydrolase, pyrrolidonyl arylamidase, leucine aminopeptidase, acetoin (Voges–Proskauer test) and β-galactosidase. Does not produce alkaline phosphatase, α-galactosidase and β-glucuronidase. Hippurate hydrolysis is negative; aesculin hydrolysis is positive. Acid is produced from glycerol, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-trehalose and gentiobiose. Acid is not produced from erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-melibiose, sucrose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. Acid production from L-arabinose (strain W213 is weakly positive in API 50 CH kit, but negative using API 20 Strep; strain W442^T is negative) and gluconate (strain W213 is positive, W442^T is negative) is variable. The G+C content of strains W213 and W442^T is 35·6 and 36·7 mol%, respectively.

The type strain, W442^T (=CCM 7319^T=LMG 23085^T), and the other strain, W213 (=CCM 7318=LMG 23084), were isolated from surface waters.

Description of *Enterococcus termitis* sp. nov.

Enterococcus termitis (ter.mi′tis. L. n. *termes -itis* a worm that eats wood, a woodworm, and in zoology the name of a scientific genus; L. gen. n. *termitis* of a termite).

Cells are Gram-positive, ovoid cocci, occurring in pairs, short chains or small groups. They elongate in the direction of the chains. Colonies on Columbia agar supplemented with sheep blood are non-pigmented, shiny, circular, smooth with entire margins and about 1 mm in diameter after 24 h of cultivation at 37 °C. The type strain of the species grows well on Todd–Hewitt agar and BHI agar; growth on MRS medium is less abundant. Poor growth on Slanetz–Bartley medium containing 0·04 % sodium azide in small dark-red colonies. Positive growth with positive aesculin reaction on kanamycin/aesculin/azide agar and bile/aesculin agar. Non-motile. Growth occurs in BHI broth at 10–45 °C, pH 9·6 and in the presence of 6·5 % NaCl. Catalase reaction is negative on blood-containing as well as on blood-free media. Produces leucine aminopeptidase. Does not produce pyrrolidonyl arylamidase, arginine dihydrolase, acetoin (Voges–Proskauer test), α-galactosidase, β-galactosidase, β-glucuronidase or alkaline phosphatase. Hippurate hydrolysis is negative; aesculin hydrolysis is positive. Acid is produced from glycerol, ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-trehalose, gentiobiose and gluconate. Acid is not produced from erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, D-melibiose, sucrose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. The G+C content of the type strain is 37·1 mol%.

The type strain, LMG 8895^T(=CCM 7300^T), originated from the gut of a termite.

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