Enterococcus rivorum sp. nov., from water of pristine brooks

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A significant number of *Enterococcus* strains from pristine waters of two brooks in Finland formed a distinct cluster on the basis of whole-cell protein fingerprinting by one-dimensional SDS-PAGE. The strains shared the following characteristics. Cells were ovoid, Gram-positive-staining and non-spore-forming, appearing singly or in pairs or chains. They were facultatively anaerobic and catalase-negative. Growth in broth containing 6.5 % NaCl or at 45 °C was weak or absent. Production of D antigen was variable. The strains tolerated 60 °C for 30 min, 40 % bile and tellurite, hydrolysed aesculin strongly and gelatin weakly, produced no acid from hippurate and did not reduce it, grew weakly at 10 °C, showed a strong reaction for the Voges–Proskauer test and produced acid from methyl α -D-glucoside, mannitol, sorbitol and sucrose, with weak or no production of acid from methyl α -D-mannoside, L-arabinose, gluconate and L-xylose. Several of the strains were selected for identification on the basis of sequencing of almost the whole 16S rRNA gene and partial *atpA* and *pheS* genes and of (GTG)₅-PCR fingerprints. Partial *atpA* and *pheS* gene sequencing was also performed for those type strains of *Enterococcus* species without available sequences in the database. The pristine brook isolates formed a novel species, for which the name *Enterococcus rivorum* sp. nov. (type strain S299^T = HAMB1 3055^T = LMG 25899^T = CCM 7986^T) is proposed. On the basis of 16S rRNA gene sequence similarity, *E. rivorum* sp. nov. is related to the *Enterococcus faecalis* genogroup. It is distinguished from described *Enterococcus* species on the basis of 16S rRNA, *atpA* and *pheS* gene sequences and whole-cell protein and (GTG)₅-PCR fingerprints. It is most closely related to *E. faecalis*, but DNA–DNA hybridization confirms it to represent a novel species.

The genus *Enterococcus* includes human and animal intestinal commensals, but also increasingly problematic pathogens (Švec & Devriese, 2009). Enterococci have been used as faecal indicators in hygienic water analysis, but the common occurrence of isolates that remain unidentified has caused uncertainty in interpretation (Clausen *et al.*, 1977). Recent developments in taxonomy (Švec & Devriese, 2009) offer a better basis to reveal natural environments for

each species and to estimate the value of each species as an indicator of water contamination.

Morphological and physiological characteristics are used to confirm that isolates belong to the genus *Enterococcus*, but identification to the species level is often uncertain (Švec & Devriese, 2009). Differentiation of *Enterococcus* species on the basis of 16S rRNA gene sequencing has not been clear enough for all the closely related strains that have been described. Recently, novel enterococcal species have been differentiated by using more variable gene fragments, namely the α -subunit of ATP synthase (*atpA*) (Naser *et al.*, 2005a) and phenylalanyl-tRNA synthase (*pheS*) (Švec *et al.*, 2005). Whole-cell protein fingerprints, which reveal an

The GenBank/EMBL/DDBJ accession numbers for the sequences obtained in this study are detailed in Table S1.

A supplementary table and four supplementary figures are available with the online version of this paper.

array of primary expression products of protein-encoding genes, have proved to be efficient in the differentiation of *Enterococcus* species (Niemi *et al.*, 1993; Carvalho *et al.*, 2006; Švec & Devriese, 2009).

The species composition of enterococci in pristine waters in Finland remained obscure even when water samples with faecal contamination yielded known species (Niemi *et al.*, 1993). The aim of the present study was to clarify the taxonomic position of the previously unidentified pristine water enterococci, which formed 32 % of pristine water isolates (the sole habitat detected) observed as whole-cell protein fingerprint cluster VII, which also displayed low maximum growth temperatures (Niemi *et al.*, 1993), by 16S rRNA, *atpA* and *pheS* sequencing and (GTG)₅-PCR and whole-cell protein fingerprinting.

Enterococci were isolated from pristine water bodies (no human habitation, agriculture or industry in the catchment area) in Finland on KF Streptococcus agar (Difco) using incubation at 35 ± 2 °C for 44 ± 4 h and were characterized as described by Niemi *et al.* (1993) for the Skerman criteria (growth at 10 and 45 °C, tolerance of 6.5 % NaCl and K₂TeO₃, tolerance of 60 °C for 30 min) according to Facklam & Wilkinson (1981), for other physiological traits (API 50CHS), the presence of D antigen and for maximum growth temperature. Inocula and cell biomass were cultivated in brain heart infusion (BHI) broth (Difco), yielding good growth at 35–37 °C overnight. Isolates were stored in skimmed milk tubes at –70 °C. Analysed strains isolated from a brook from Pond Kilpilampi (WGS84lat 6051.44 and WGS84long 2401.31), S298, S299 and S309, and from a brook from Pond Vaunulampi (WGS84lat 6046.26 and WGS84long 2404.68), S259, S262, S271 and S280, are listed in Table S1 (available in IJSEM Online) together with the type strains subjected to sequencing in the present study. Reference *Enterococcus* species type strains, including that of the type species of the genus *Enterococcus faecalis* (CCM 7000^T = DSM 20478^T), that were included in individual experiments were obtained from the BCCM/LMG (<http://bccm.belspo.be/about/lmg.php>) and CCM (<http://www.sci.muni.cz/ccm/>) bacteria collections.

DNA was isolated from bacterial cells using a Fast DNA kit and FastPrep Instrument (Q-BIOgene) according to the manufacturer's instructions but with an additional enzymic pretreatment: the pellet of cells was suspended in a mixture of 100 µl phosphate buffer, 3 µl lysozyme solution (1000 U in 1 ml phosphate buffer) and 2.2 µl mutanolysin solution (5000 U in 1 ml buffer at pH 6.2 containing 0.1 mol KH₂PO₄ l^{–1}) and incubated for 45 min at room temperature.

A sequence of about 1585 bp of the 16S rRNA gene was amplified as described by Weisburg *et al.* (1991) using the forward primer fD1 (5'-CCGAATTCGTCGACAACAGAG-TTTGATCCTGGCTCA-3') and reverse primer rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'). A 1102 bp fragment of the *atpA* gene (encoding the

α-subunit of ATP synthase) was amplified using primers atpA-20-F, atpA-22-F, atpA-23-R, atpA-24-F, atpA-25-R and atpA-27-R according to Naser *et al.* (2005a). A 455 bp fragment of the *pheS* gene (encoding phenylalanyl-tRNA synthase) was amplified using primers pheS-21-F, pheS-22-R and pheS-23-R according to Švec *et al.* (2005). In addition to our environmental isolates, the type strains of *Enterococcus caccae*, *E. camelliae*, *E. devriesei*, *E. silesiacus*, *E. termitis* and *E. thailandicus* were sequenced for the *atpA* and *pheS* genes (Table S1).

The amplified fragments were purified using a Montage PCR Cleanup kit. Sequencing reactions were done with both forward and reverse primers using BigDye Chemistry and the reactions were analysed on an ABI 3130 DNA Sequencer. In addition, for sequencing of the 16S rRNA gene, internal primers pC, pC', pD, pD', pE' and pF were used (Edwards *et al.*, 1989). For strain S271, two amplification products (fD1–pE' and pD–rD1) were produced for 16S rRNA gene sequencing. The sequences obtained were edited and assembled using the Gap4 program from the Staden package (Staden *et al.*, 2000).

BLASTN searches were run using version 2.2.24+ (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the GenBank + EMBL + DDBJ + PDB sequence database. MAFFT version 6.8 (Katoh & Toh, 2008) was used for sequence alignment of *pheS* and *atpA*. The Ribosomal Database Project aligner was used for the alignment of 16S rRNA gene sequences (Cole *et al.*, 2009). Alignments were edited manually and all unknown bases were removed from the analysis. Maximum-likelihood trees with 1000 bootstrap resamplings with branch lengths were drawn with RAXML version 7.0.3 (Stamatakis, 2006) on 16S rRNA gene sequences of about 1250 bp.

Repetitive sequence-based PCR fingerprinting with the (GTG)₅ primer was performed as described by Švec *et al.* (2008). Briefly, 1 µl bacterial DNA isolated by an alkaline extraction procedure and 24 µl PCR mixture were included in PCRs performed in a Tpersonal thermocycler using PCR conditions described previously (Švec *et al.*, 2008). PCR products were separated in 1.5 % agarose gels (20 × 20 cm) for 16 h at 60 V (approx. 1.7 V cm^{–1}) in 0.5 × TBE buffer. Fingerprints were digitized and processed using the BioNumerics 6.5 software and compared to the in-house CCM database containing multiple reference strains covering all hitherto-described *Enterococcus* species. Numerical analysis of the (GTG)₅-PCR fingerprints was conducted with Pearson's correlation coefficients and the resulting dendrogram was reconstructed using the UPGMA clustering method.

SDS-PAGE of whole-cell proteins was performed according to Pot *et al.* (1994). Cultures were grown at 37 °C for 24 h on BHI agar. The cells were disrupted with a MagNA Lyser instrument. SigmaMarker Wide Range (6.5–205 kDa) was used as a molecular mass marker set. Numerical analysis of digitized protein profiles was carried out with the BioNumerics 6.5 software using Pearson's correlation similarity

Table 1. Distinguishing characteristics of the novel strains and *E. faecalis* genogroup species

Strains/species: 1, S299^T; 2, S259; 3, S262; 4, S271; 5, S280; 6, S298; 7, S309; 8, *E. caccae* (data from Carvalho *et al.*, 2006); 9, *E. silesiacus* (Švec *et al.*, 2006); 10, *E. haemoperoxidus* (Švec *et al.*, 2001); 11, *E. moraviensis* (Švec *et al.*, 2001); 12, *E. termitis* (Švec *et al.*, 2006); 13, *E. faecalis* (Schleifer & Kilpper-Bälz, 1984); 14, *E. faecalis* DSM 20478^T (data from the present study). +, Positive; v, variation between strains; w, weak; d, delayed; –, negative; ND, no data available/not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group D antigen	–	–	–	+	–	+	+	+	ND	+	+	ND	+	ND
Growth at 45 °C	–	w	w	w	w	–	–	+	–	–	–	+	+	+
Tolerance of tellurite	+	+	+	+	+	+	+	–	ND	–	–	ND	+	+
Growth in 6.5 % (w/v) NaCl	–	w	w	–	–	–	–	+	+	+	+	+	+	+
Hydrolysis of hippurate	–	–	–	–	–	–	–	v	–	+	+	–	+	–
Hydrolysis of arginine	–	–	–	+	–	–	–	–	+	+/d	–	–	+	+
Voges–Proskauer reaction	+	+	+	+	+	+	+	+	+	+	+	–	+	+
Acid production from:														
Methyl α-D-glucoside	+	+	+	+	+	+	+	ND	–	+	+	+	–	–
Methyl α-D-mannoside	–	–	–	–	–	–	–	ND	–	–	–	+	–	–
L-Arabinose	–	–	–	w	–	–	–	–	v	–	+	–	–	–
Gluconate	–	–	–	w	–	–	–	ND	v	–	–	+	v	+
Mannitol	+	+	+	+	+	+	+	–	–	v	+	–	+	+
Sorbitol	+	+	+	+	+	+	+	–	–	–	–	ND	v	+
Sucrose	+	+	+	+	+	+	+	+	–	+	+	–	+	+
L-Xylose	–	–	–	w	–	–	–	ND	–	–	–	–	–	–

Description of *Enterococcus rivorum* sp. nov.

Enterococcus rivorum (ri.vo'rum. L. gen. pl. n. *rivorum* of brooks).

Cells are ovoid and Gram-stain-positive and occur singly or in pairs or chains. Non-spore-forming. White colonies are formed on BHI agar at 35 °C. Strains are facultatively anaerobic and catalase-negative. Growth in broth containing 6.5 % NaCl and at 45 °C is weak or absent and production of D antigen is variable. The maximum temperature for growth on KF Streptococcus agar is 36.5–39.6 °C. Strains tolerate 60 °C for 30 min, tolerate 40 % bile, tolerate and reduce tellurite, hydrolyse aesculin strongly and gelatin weakly and produce no acid from hippurate and do not reduce it. Generally weak growth occurs at 10 °C. Strains exhibit a strong positive reaction for acid production from *N*-acetylglucosamine and the Voges–Proskauer test and produce acid from glycerol, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, methyl α-D-glucoside, amygdalin, arbutin, aesculin (API 50 CHS), salicin, cellobiose, maltose, sucrose, trehalose, starch and gentiobiose. Variable for hydrolysis of arginine and variable production of acid occurs from D-xylose, inositol, lactose, melezitose, xylitol, turanose, D-lyxose, D-tagatose and D-arabitol. Strains display weak or no production of acid from D- and L-arabinose, L-xylose, gluconate and 5-ketogluconate and produce no acid from erythritol, adonitol, methyl β-xyloside, L-sorbose, rhamnose, dulcitol, methyl α-D-mannoside, melibiose, inulin, raffinose, glycogen, D- or L-fucose, L-arabitol or 2-ketogluconate.

The type strain, S299^T (=HAMBI 3055^T =LMG 25899^T =CCM 7986^T), was isolated by one of the authors

(R. M. N.) from a pristine brook in Finland in 1990. It is positive for acid production from D-xylose, weakly positive for acid production from inositol, melezitose and turanose and negative for production of D antigen and acid production from lactose, xylitol, D-lyxose, D-tagatose and D-arabitol.

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