Enterococcus plantarum sp. nov., isolated from plants

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Eight Gram-positive, catalase-negative bacterial strains were isolated during screening of enterococcal populations on plants. rep-PCR fingerprinting using the (GTG)₅ primer showed that the isolates constituted a single cluster that was separate from all known enterococcal species. 16S rRNA gene sequence phylogenetic analysis of three representative strains showed that the isolates belonged to the genus *Enterococcus* and that they clustered with the *Enterococcus faecalis* species group. Sequencing of the genes for the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and the RNA polymerase alpha subunit (*rpoA*) also revealed the isolates' separate taxonomic position. Application of whole-cell protein fingerprinting, automated ribotyping and extensive phenotyping demonstrated the genetic and phenotypic homogeneity of the isolates and confirmed their separate position within the *E. faecalis* species group. The isolates represent a novel species of the genus *Enterococcus*, for which the name *Enterococcus plantarum* sp. nov. is proposed; the type strain is CCM 7889^T (=LMG 26214^T=C27^T).

The genus *Enterococcus* is phylogenetically placed in the family *Enterococcaceae* within the order *Lactobacillales*. The genus currently comprises 35 species (Euzéby, 1997) and occurs in the human and animal gastrointestinal tract, food and the environment. Enterococci are well-known opportunistic pathogens and are important to human medicine as they are a common cause of a variety of infections, including nosocomial infections. In contrast, they play a beneficial role in many dairy and fermented foods and certain strains are used as probiotics (Franz & Holzapfel, 2004; Švec & Devriese, 2009). The environmental enterococci are a taxonomically diverse group. Particularly, the enterococci associated with plants represent lesser-known bacterial groups and only a handful of taxonomic studies dealing with enterococci isolated from plants have been published to date. Mundt (1961, 1963) who studied the occurrence of enterococci (group D streptococci) on agricultural plants and grasses concluded that they are

temporary residents and inhabit plants because of the wind and insect activity. Later studies, conducted by Ulrich & Müller (1998), Müller *et al.* (2001) and Ott *et al.* (2001), revealed the occurrence of *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus casseliflavus* and *Enterococcus sulfureus* on plants; however, these works reported a high proportion of enterococcal strains as representing unknown plant-associated species.

Eight bacterial strains were isolated during a project dealing with the enterococcal microflora of plants. The isolates were retrieved from different plants from June to October 2009 (Table 1). Plant samples were obtained from a meadow situated about 600 m from the village of Lipina in the Olomouc district in the Czech Republic (49° 44' 29.979" N 17° 19' 24.696" E). The meadow is surrounded by a mixed forest, is not influenced by farm animals and is exploited only for hay harvesting. Plant specimens were aseptically sampled and initially cultivated in brain-heart infusion broth (BHI; Oxoid) at 37 °C for 48 h and subsequently grown on kanamycin aesculin azide agar (KAA; Merck) at 37 °C for 48 h. Individual colonies of the typical black enterococcal colony morphology were purified on BHI agar and stored at –70 °C. Reference strains were deposited in the Czech Collection of Microorganisms (CCM; Masaryk University, Brno, Czech Republic; <http://www.sci.muni.cz/ccm/>) and in the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium; <http://bccm.belspo.be>).

Abbreviations: *pheS*, phenylalanyl-tRNA synthase alpha subunit gene; *rpoA*, RNA polymerase alpha subunit gene.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CCM 7889^T, CCM 7887 and CCM 7890 are HQ847537–HQ847539, respectively. The GenBank/EMBL/DDBJ accession numbers for the *pheS* and *rpoA* sequences obtained in this study are HQ847540–HQ847551 and JF276437–JF276444, respectively.

Four supplementary figures and a supplementary table are available with the online version of this paper.

Table 1. Sources and isolation dates of strains

CCM, Czech Collection of Microorganisms, Czech Republic; LMG, BCCM/LMG Bacteria Collection, Belgium.

Strain number	Other strain designations	Source	Isolation date
CCM 7887	C38	<i>Aegopodium</i> sp., flower	24 June 2009
CCM 7888	G25	<i>Campanula</i> sp., flower	12 October 2009
CCM 7889 ^T	C27 ^T , LMG 26214 ^T	<i>Aegopodium</i> sp., flower	24 June 2009
CCM 7890	D15	<i>Melampyrum</i> sp., flower	31 July 2009
P3745	B4	<i>Carex</i> sp., flower	1 June 2009
P3746	B29, LMG 26212	<i>Carduus</i> sp., flower	1 June 2009
P3747	D27/2, LMG 26213	<i>Thymus</i> sp., complete plant	31 July 2009
P3748	G10/2, LMG 26211	<i>Leontodon</i> sp., flower	12 October 2009

Initial genotypic screening was performed using rep-PCR fingerprinting with the (GTG)₅ primer, which has been evaluated for the identification of species of the genus *Enterococcus* (Švec *et al.*, 2005). The method was performed as described by Švec *et al.* (2008). Numerical analysis of the (GTG)₅-PCR fingerprints was done using BioNumerics version 6.5 (Applied Maths) and resulting fingerprints were compared with those in the in-house CCM database, which contains multiple strains of all hitherto-described species of the genus *Enterococcus*. Fig. S1 (available in IJSEM Online) shows the (GTG)₅-PCR fingerprints obtained from the isolates and demonstrates their separation from members of the genus *Enterococcus*. All eight isolates were placed in a single cluster that was clearly separate from the reference strains. The cluster contained two subclusters that showed differences in bands in the range of 1600–1800 bp.

For 16S rRNA gene sequencing, strains CCM 7887, CCM 7889^T and CCM 7890 were cultivated on BHI agar at 37 °C overnight. DNA was extracted by suspending one loop of cells in 500 µl sterile deionized water and heating at 80 °C for 20 min. Subsequently, the crude extracts were centrifuged at 14 000 r.p.m. for 1 min and the supernatant was used directly as a template for PCR. Amplification was performed using the universal primers 16S_F1 (5'-AGAGTTTGATCCTGGCTCAG-3'; Coenye *et al.*, 1999) and 16S_R1530 (5'-AAGGAGGTGATCCAGCCGCA-3'; Hughes *et al.*, 2000). The PCR programme consisted of: denaturation for 5 min at 95 °C; three cycles of 30 s at 95 °C, 60 s at 58 °C and 60 s at 72 °C; 30 cycles of 20 s at 95 °C, 30 s at 58 °C and 75 s at 72 °C; and final extension for 7 min at 72 °C. The PCR products were subsequently purified using a High Pure PCR product purification kit (Roche Diagnostics). Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). Partial 16S rRNA gene sequences were combined into a single consensus sequence using the internet assembler tool CAP3 Sequence Assembly Program (Huang & Madan, 1999). The sequences obtained and reference sequences downloaded from GenBank were aligned using CLUSTAL W (Thompson *et al.*, 1994) with a gap opening penalty of 15 and a gap extension penalty of 6.66. Evolutionary distances were computed by maximum composite likelihood and a

phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using MEGA4 (Tamura *et al.*, 2007). The tree topology was evaluated and confirmed by maximum parsimony using Bionumerics version 6.5. Fig. 1 is the resulting consensus tree inferred from 1000 replicates and shows the phylogenetic position of strains CCM 7887, CCM 7889^T and CCM 7890 within the genus *Enterococcus*. All isolates revealed 100 % 16S rRNA gene sequence similarity with each other and were placed within the *E. faecalis* species group, which contained *E. faecalis* JCM 5803^T, *Enterococcus caccae* 2215-02^T, *Enterococcus haemoperoxidus* CCM 4851^T, *Enterococcus moraviensis* CCM 4856^T, *Enterococcus silesiacus* LMG 23085^T and *Enterococcus termitis* LMG 8895^T. Global alignment performed by the algorithm of Myers & Miller (1988) using the EzTaxon web-based tool (Chun *et al.*, 2007) showed that the isolates shared 99.9 % 16S rRNA gene sequence similarity with *E. moraviensis* CCM 4856^T and 99.8 % 16S rRNA gene sequence similarity with *E. silesiacus* LMG 23085^T and *E. haemoperoxidus* CCM 4851^T.

Multilocus sequence analysis based on partial sequences for the genes of the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and the RNA polymerase alpha subunit (*rpoA*) was performed for all isolates, according to the method described by Naser *et al.* (2005), who demonstrated that these genes are valuable tools for the delineation of enterococci species. Chromosomal DNA was isolated from overnight cultures grown on BHI agar at 37 °C and extracted as described above for 16S rRNA gene sequence. Primer sequences and PCR amplification conditions were as described by Naser *et al.* (2005). The amplified *pheS* and *rpoA* PCR products were subsequently purified using the High Pure PCR product purification kit (Roche Diagnostics) and the sequencing procedure was performed by Eurofins MWG Operon (Ebersberg, Germany). The *pheS* and *rpoA* sequences of the type strains representing the currently validly described *Enterococcus* spp. were retrieved from the GenBank database. The sequences were aligned in CLUSTAL W (Thompson *et al.*, 1994) using the gap opening penalty 15 and gap extension penalty 6.66 parameters and the phylogenetic tree was calculated by the neighbour-joining method (Saitou & Nei, 1987) using MEGA4 (Tamura *et al.*, 2007). The resulting *pheS* and *rpoA* dendrograms inferred

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains CCM 7889^T, CCM 7887 and CCM 7890 within the genus *Enterococcus*. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 1% sequence divergence.

from 1000 replicates (Fig. 2) demonstrated that the isolates formed a homogeneous cluster (>99.7 and >99.8% *pheS* and *rpoA* gene sequence similarities, respectively) that was clearly separate from members of the *E. faecalis* species group. The isolates showed the highest *pheS* sequence similarity (88.1%) with *E. haemoperoxidus* LMG 19487^T and the highest *rpoA* sequence similarity (97.6%) with *E. silesiacus* CCM 7319^T and *E. caccae* 7399^T. Extended *pheS* and *rpoA* phylogenetic trees for the isolates and the type strains of all species of the genus *Enterococcus* are shown in Figs S2 and S3, respectively.

Phenotypic characterization was performed using the API 20 Strep and API 50 CH systems (bioMérieux), STREPTOtest 24 (Pliva-Lachema Diagnostika) and GP2 MicroPlates (Biolog), as well as using conventional growth and physiological tests. Growth characteristics were tested on Columbia agar (Oxoid) supplemented with 5% sheep blood, BHI agar, KAA agar and Slanetz and Bartley medium (Oxoid). Growth at 10 and 45 °C, with 6.5% NaCl and at pH 9.6 was examined in BHI broth for up to 5 days.

Catalase production was tested using an ID Colour Catalase kit (bioMérieux). Lancefield antigens were determined using a Strep kit (Diamond). Production of gas from glucose was tested according to Sperber & Swan (1976). Pyrrolidonyl arylamidase and acetoin production were tested using PYRAtest and VPtest, respectively (PLIVA-Lachema Diagnostika). Urease production was tested on Urea Agar Base Christensen (HiMedia). Motility was studied on stab-inoculated semi-solid medium [1% tryptone (Oxoid), 0.4% nutrient broth (Merck), 0.5% NaCl and 0.4% agar (Sigma-Aldrich)] with cultivation at 37 °C for 24 h and then at room temperature for up to 5 days. The results are given in the species description and Table S1. The isolates could be differentiated from members of the *E. faecalis* species group by the characteristics given in Table 2.

Whole-cell protein fingerprints have been proved to correlate well with DNA–DNA relatedness (Vandamme *et al.*, 1996) and have been evaluated for the identification of enterococci and the description of novel *Enterococcus* species in numerous studies (Alves *et al.*, 2004; Facklam

Fig. 2. Neighbour-joining phylogenetic trees based on *pheS* (a) and *rpoA* (b) sequences showing the positions of *Enterococcus plantarum* sp. nov. within the *E. faecalis* species group. Bootstrap values ($\geq 50\%$) based on 1000 replications are shown at branch nodes. *E. faecium* LMG 11423^T was used as an outgroup. Bars, 5% sequence divergence.

et al., 2002; Merquior *et al.*, 1994). The present study used this method to confirm the separation of the isolates from members of the *E. faecalis* species group. Whole-cell protein profiling was performed according to the protocol described by Pot *et al.* (1994). Briefly, the isolates were grown on BHI agar at 37 °C for 24 h. Harvested cells were disrupted using a MagNA Lyser (Roche). SigmaMarker Wide Range (6.5–205 kDa; Sigma) was used as a molecular mass marker. Numerical analysis of digitized protein profiles was done using BioNumerics version 6.5. A dendrogram was constructed using Pearson's correlation similarity coefficient with the unweighted pair group method using arithmetic averages (UPGMA). All eight isolates constituted a single cluster and showed nearly identical protein profiles (88.7% similarity), which confirmed that they represented a single species. The most similar protein profile (75.6% similarity) to those of the isolates was that of *E. silesiacus* CCM 7319^T. As shown in Fig. 3, the whole-cell protein fingerprinting clearly separated all eight isolates from members of the *E. faecalis* species group.

Automated ribotyping with the restriction enzyme *EcoRI* was performed using the RiboPrinter microbial characterization system (DuPont Qualicon) in accordance with the standard protocol for lactic acid bacteria provided by the manufacturer. Bacterial cells used for ribotyping were cultivated on BHI agar at 37 °C for 24 h. The obtained ribopatterns were normalized and automatically categorized into ribogroups using the reference database DUP 2008 and the RiboExplorer version 2.1.4216.0 operating software (DuPont Qualicon). A dendrogram was calculated using Pearson's correlation coefficients with UPGMA using Bionumerics version 6.5. The ribopatterns were imported into the Bionumerics software using the load samples import script obtained from the manufacturer. The isolates revealed nearly identical fingerprint patterns (92.8% similarity) and were clearly separated from members of the *E. faecalis* species group. The most similar ribotype pattern (51.8% similarity) to those of the isolates was that of *E. silesiacus* CCM 7319^T. Fig. S4 shows ribotype patterns obtained from the isolates and members of the *E. faecalis* species group.

Table 2. Phenotypic tests differentiating *Enterococcus plantarum* sp. nov. from its closest phylogenetic relatives

Species: 1, *Enterococcus plantarum* sp. nov.; 2, *E. caccae*; 3, *E. faecalis*; 4, *E. haemoperoxidus*; 5, *E. moraviensis*; 6, *E. silesiacus*; 7, *E. termitis*. Data were taken from this study and Švec & Devriese (2009). +, Positive; v, variable; –, negative.

Characteristic	1	2	3	4	5	6	7
Production of:							
Acetoin	+	+	+	+	+	+	–
Arginine dihydrolase	–	–	+	+	–	+	–
Pyrrolidonyl arylamidase	+	+	+	+	v	+	–
β -Galactosidase	–	–	–	–	v	+	–
Hippurate hydrolysis	+	+	+	+	+	–	–
Production of acid from:							
L-Arabinose	–	–	–	–	+	v	–
Glycerol	–	+	+	+	+	+	+
Lactose	–	–	+	+	+	+	+
Melezitose	+	–	v	+	+	–	–
Methyl α -D-glucopyranoside	–	+	–	+	+	–	+
Sucrose	+	+	+	+	+	–	–
D-Tagatose	–	+	+	–	+	–	–

For the determination of DNA G+C content, DNA from strains P3748 and CCM 7889^T was prepared according to the procedure described by Vancanneyt *et al.* (2001). The

G+C content was determined by enzymically degrading the DNA into nucleosides as described by Mesbah & Whitman (1989). The obtained nucleoside mixture was then separated by HPLC using a Waters Symmetry Shield C8 column at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) and 1.5 % (v/v) acetonitrile. Non-methylated phage λ DNA (Sigma) was used as the calibration reference. The DNA G+C content of strains P3748 and CCM 7889^T was 36 mol%.

In summary, the results obtained in the present study demonstrated that the eight enterococcal isolates originating from plants represent a genotypically and phenotypically coherent taxon that can be distinguished from established enterococcal species. We therefore propose to classify them in a novel species, *Enterococcus plantarum* sp. nov.

Description of *Enterococcus plantarum* sp. nov.

Enterococcus plantarum (plan.ta'rum. L. n. *planta* a sprout; L. gen. pl. n. *plantarum* of plants).

The species description is based on eight isolates. Cells are Gram-positive, non-motile, ovoid cocci or short rods, occurring in pairs, short chains or small groups. Cocci are elongated in the direction of the chains. On Columbia agar supplemented with 5 % sheep blood and on BHI agar, colonies are yellowish, smooth and circular with entire

Fig. 3. Dendrogram based on whole-cell protein profiles of *Enterococcus plantarum* sp. nov. and members of the *E. faecalis* species group. The dendrogram was calculated using Pearson's correlation coefficients with the UPGMA clustering method (*r*, expressed as percentage similarity values).

margins (1 mm in diameter after cultivation at 37 °C for 24 h). Grows on KAA agar with positive aesculin reaction and grows weakly on Slanetz and Bartley medium with tiny dark-red colonies. Strongly catalase-positive on blood agar; catalase-negative on blood-free media. Production of gas from glucose is negative. Grows at 10 °C and 45 °C, at pH 9.6 and with 6.5 % NaCl. Group D antigen-positive. Produces acetoin (Voges–Proskauer test), leucine arylamidase, pyrrolidonyl arylamidase, urease, β -mannosidase and β -glucosidase, but not alkaline phosphatase, arginine dihydrolase, α - or β -galactosidase, β -glucuronidase or α -methylglucosidase. Production of *N*-acetylglucosaminidase is negative except for strain P3747. Aesculin and hippurate hydrolysis is positive. Produces acid from *N*-acetylglucosamine, amygdalin, arbutin, cellobiose, D-fructose, β -gentiobiose, D-glucose, D-mannose, melezitose, D-ribose, salicin, sucrose and trehalose, but not from D-adonitol, starch, D- or L-arabinose, D- or L-arabitol, dulcitol, erythritol, D- or L-fucose, gluconate, glycerol, glycogen, inositol, inulin, 2-ketogluconate, 5-ketogluconate, lactose, D-lyxose, D-mannitol, melibiose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, methyl β -D-xylopyranoside, raffinose, L-rhamnose, pullulan, D-sorbitol, L-sorbose, D-tagatose, turanose, xylitol or D- or L-xylose. Production of acid from D-galactose is negative except for strain CCM 7890. Production of acid from maltose is strain dependent (positive for strains CCM 7887, CCM 7889^T, CCM 7890, P3745 and P3747). With GP MicroPlates, utilizes *N*-acetyl-D-glucosamine, arbutin, cellobiose, D-fructose, α -D-glucose, D-mannose, methyl β -D-glucoside, salicin, sucrose, trehalose, glycerol, inosine, thymidine and uridine, but not α - or β -cyclodextrin, glycogen, inulin, mannan, Tweens 40 or 80, *N*-acetyl- β -D-mannosamine, L-arabinose, D-arabitol, L-fucose, D-galactose, D-galacturonic acid, D-gluconic acid, α -lactose, lactulose, maltotriose, melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methyl glucose, methyl α -D-mannoside, raffinose, L-rhamnose, sedoheptulosan, D-sorbitol, stachyose, D-tagatose, xylitol, D-xylose, acetic acid, α -, β - or γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -keto-glutaric acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- or 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- or L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, AMP, TMP, UMP, D-fructose 6-phosphate, α -D-glucose 1-phosphate, D-glucose 6-phosphate or DL- α -glycerol phosphate. Table S1 gives the strain-dependent results with GP MicroPlates. The DNA G + C content of strains CCM 7889^T and P3748 is 36 mol%.

The type strain, CCM 7889^T (=LMG 26214^T=C27^T), was isolated from flowers of *Aegopodium* sp.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (project MSM0021622416).

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