Enterococcus olivae sp. nov., isolated from Spanish-style green-olive fermentations

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Six strains of a hitherto unknown, Gram-stain-positive coccus were recovered from samples of Spanish-style green-olive fermentations. The 16S rRNA gene sequences from these isolates shared 98.7 % and 98.5 % of their nucleotide positions with those from *Enterococcus saccharolyticus* subsp. *taiwanensis* 812^T and from *E. saccharolyticus* subsp. *saccharolyticus* ATCC 43076^T, respectively. The sequence of the *rpoA* gene in the isolates was 95 % similar to that of *E. saccharolyticus* CECT 4309^T (=ATCC 43076^T). The 16S rRNA and *rpoA* gene phylogenies revealed that the isolates grouped in a statistically well-supported cluster separate from *E. saccharolyticus*. Enzyme activity profiles as well as fermentation patterns differentiated the novel bacteria from other members of the *Enterococcus* genus. Finally, phenotypic, genotypic and phylogenetic data supported the identification of a novel species of the genus *Enterococcus*, for which the name *Enterococcus olivae* sp. nov. is proposed. The type strain is IGG16.11^T (=CECT 8063^T=DSM 25431^T).

Enterococci are Gram-positive cocci belonging to the lactic acid bacteria (LAB) group and are ubiquitous in nature. They are commensal inhabitants of the gastrointestinal tract of mammalians and are also found in fermented foods, dairy products, plants, soil or water (Baele *et al.*, 2000; Devriese *et al.*, 1992; Devriese & Pot, 1995; Franz *et al.*, 1999; Klein, 2003). Enterococci can be involved in human nosocomial infections (Teixeira & Facklam, 2003), but they are also considered beneficial and safe in fermented products (Giraffa, 2002). At the time of this writing, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN; http://www.bacterio.net), the genus *Enterococcus* consisted of 53 species and 2 subspecies.

Spanish-style green-olive fermentations appear to be dominated by *Lactobacillus pentosus* strains (Ruiz-Barba & Jiménez-Díaz, 2012), but enterococci seem to have a role at the crucial initial stage of these fermentations because of their tolerance to high pH and their LAB characteristics (De Castro *et al.*, 2002; Corsetti *et al.*, 2012). Enterococci isolated from olive fermentations mostly belong to the *Enterococcus casseliflavus* group (De Castro *et al.*, 2002; De Bellis *et al.*, 2010).

During the characterization of the microbiota associated to Spanish-style green-olive fermentations in two large table-olive manufacturing companies (4,000 to 8,000 tonnes of olives handled per season) in the province of Sevilla, south-western Spain, samples of the fermenting brines of ten 10-tonne fermenters were taken along the different fermentation stages at each fermentation yard (data not shown). These samples were analysed through culture-dependent techniques. The samples were serially diluted and spread on plates of de Man–Rogosa–Sharpe (MRS; Biokar Diagnostics) agar supplemented with 0.02 g bromophenol blue l^{-1} (AppliChem) and 0.05 % (w/v) L-cysteine (MRS-BPB; Lee & Lee, 2008), and on brain heart infusion (BHI; Biokar Diagnostics) agar supplemented with 0.05 % (w/v) L-cysteine. Plates were incubated anaerobically at 30 °C for 48 h. For anaerobic incubations, we used a DG250 Anaerobic Workstation (Don Whitley Scientific), with a gas mixture consisting of 10 % H_2, 10 % CO_2 and 80 % N_2. As a result, a total of 16 unidentified LAB isolates (fifteen from BHI and one from MRS-BPB) were detected. These isolates were present in all ten fermenters during the first fermentation stage (i.e. the first 10 days) at one of the

Abbreviations: LAB, lactic acid bacteria; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of *E. olivae* strains IGG16.11^T and IGG13.11 are JQ283454 and KJ566121, respectively. The accession numbers for the *rpoA* gene sequences of *E. olivae* strains IGG16.11^T and IGG13.11 are JQ283455 and KJ566122, respectively.

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

fermentation yards under study. The isolates were genotyped by RAPD by using the primer OPL5, following the protocol of Ruiz-Barba *et al.* (2005). The similarity of RAPD profiles was calculated using the pairwise Pearson's correlation coefficient, and the BioNumerics 6.6 (Applied Maths) software was used to reconstruct dendrograms using UPGMA analysis. Among the isolates, six different RAPD profiles were obtained (Fig. S1, available in the online Supplementary Material). One representative isolate of each RAPD profile was selected for further characterization. The study of the phenotypic and genotypic characteristics of these isolates indicated that they represented a novel species of the genus *Enterococcus*.

To determine the phylogenetic relationships of the isolates, the 16S rRNA gene was amplified with the primer pair 7 for (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1510r (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991), and both DNA strands were sequenced using the primers 7for, 785F (5′-GGATTAGATACCCBRGTAGTC-3′), mlb16 (5′-GGCTGCTGGCACGTAGTTAG-3′) (Kullen *et al.*, 2000) and 1510r. In addition, partial amplification and sequencing of 800 bp of the gene *rpoA* with primers rpoA-21-F (5′-ATGATYGARTTTGAAAAACC-3′) and rpoA-23-R (5′-ACHGTRTTRATDCCDGCRCG-3′) (Naser *et al.*, 2005) was performed.

The identification of the isolates and their phylogenetic neighbours was carried out using the 16S rRNA sequences obtained and the nucleotide BLAST (BLASTN) algorithm (Altschul *et al.*, 1997) to search a database on the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012), containing type strains with updated, validly published prokaryotic names. The global alignment algorithm (Myers & Miller, 1988) available at EzTaxon-e was used to calculate pairwise sequence similarity. BLASTN (at http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the DNA sequences of the *rpoA* gene in the isolates with those available in GenBank. DNA sequences belonging to type strains of the genus *Enterococcus* were obtained from the GenBank database (see Fig. 1). These sequences were aligned in CLUSTAL W (Thompson *et al.*, 1994) with MEGA 5 (version 5.2) software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed with the neighbour-joining method (Saitou and Nei, 1987). Bootstrapping analysis (1000 replicates) was used to evaluate the support of the groupings.

Chromosomal DNA was extracted following the method described by Cathcart (1995). The degree of DNA–DNA relatedness between strains IGG16.11^T and *Enterococcus saccharolyticus* CECT 4309^T was determined with a fluorometric method as described by Gonzalez & Saiz-Jimenez (2005). This method uses a real-time PCR thermocycler to measure the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m) via fluorescence values. In addition, the G+C content of genomic DNA of strain IGG16.11^T was determined by the fluorometric method described by Gonzalez & Saiz-Jimenez (2002). The results of both DNA–DNA relatedness and DNA G+C content were expressed as mean percentage and SD determined from three independent experiments.

The BLASTN analysis of the 16S rRNA gene sequences (1406 bp) from the six selected isolates indicated a similarity of 98.7 % with that of *E. saccharolyticus* subsp. *taiwanensis* 812^T and of 98.5 % with that of *E. saccharolyticus* subsp. *saccharolyticus* ATCC 43076^T. Similarly, BLAST analysis of the partial sequence (741 bp) of the *rpoA* gene from the isolates showed a similarity of 95.0 % with that of *E. saccharolyticus* CECT 4309^T (ATCC 43076^T). Both 16S rRNA and *rpoA* gene sequences were 100 % identical among the six isolates.

The phylogenetic analysis of the 16S rRNA sequence with the neighbour-joining method showed that the isolates IGG16.11^T and IGG13.11 formed a separate branch within the *Enterococcus gallinarum* group, with *E. saccharolyticus* ATCC 43076^T being the most closely related species (Fig. 1). Bootstrap resampling values of 70 % indicated statistically significant support for the *Enterococcus olivae* sp. nov. IGG16.11^T and IGG13.11 and *E. saccharolyticus* branch. The phylogenetic analysis of the *rpoA* gene sequence showed a clustering of *E. olivae* sp. nov. IGG16.11^T and IGG13.11 and *E. saccharolyticus*, with a bootstrap value of 99 % (Fig. S2). However, the topology of the tree was slightly different from that of the tree based on the 16S rRNA sequences.

The difference in melting temperature between genomic DNA from strain IGG16.11^T and from *E. saccharolyticus* CECT 4309^T was 7.5 °C. This value was above the 5 °C ΔT_m recommended as a cut-off point for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA relatedness results confirmed that strain IGG16.11^T represents a novel species clearly differentiated from *E. saccharolyticus*. The G+C content of genomic DNA of strain IGG16.11^T was 40.4 mol% (SD, 0.1).

Substrate utilization, the fermentation/oxidation profile, acid production and some other physiological characteristics were examined using the API 50 CHL fermentation kit (bioMérieux) after 48 h of incubation according to the manufacturer's instructions. Tests for growth at various temperatures as well as in NaCl were performed in BHI broth as described by Švec *et al.* (2001) and Devriese *et al.* (1993). The results are given in the species description below. The six analysed strains revealed similar biochemical profiles enabling separation from other closely related enterococcal species. They could be differentiated from most known enterococcal species by their inability to produce acid from ribose, which is considered to be typical for the genus *Enterococcus*, although several of the more recently described species of the genus, e.g. *Enterococcus camelliae* and *Enterococcus italicus*, are also negative for this trait. Table 1 shows phenotypic tests that are useful for the differentiation of *E. olivae* sp. nov. IGG16.11^T from their closest phylogenetic relatives, *E. italicus*, *E. camelliae*, *E. saccharolyticus* and *Enterococcus sulfureus*.

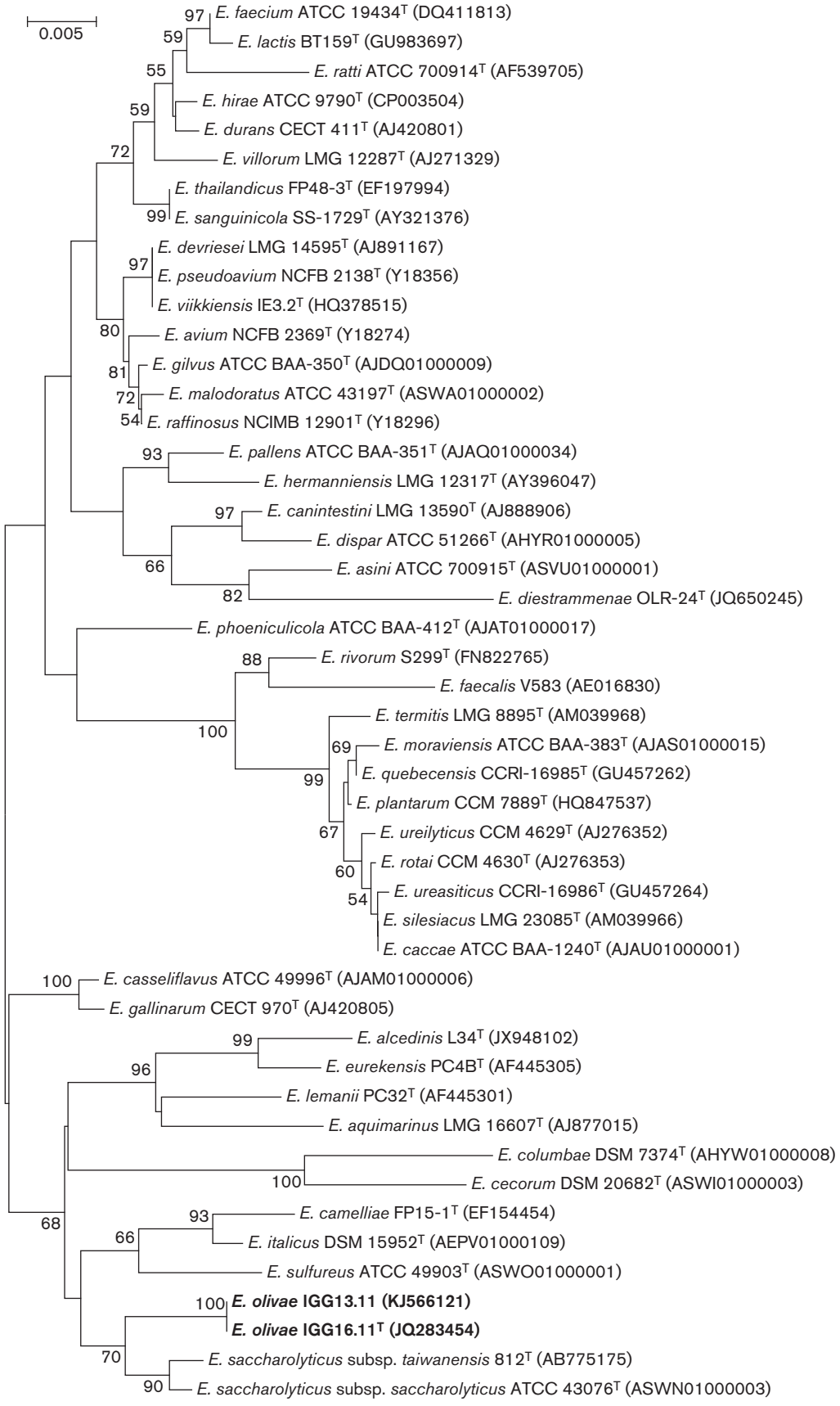
0.005
97 E. faecium ATCC 19434T (DQ411813)
59 E. lactis BT159T (GU983697)
55 E. ratti ATCC 700914T (AF539705)
59 E. hirae ATCC 9790T (CP003504)
E. durans CECT 411T (AJ420801)
72 E. villorum LMG 12287T (AJ271329)
E. thailandicus FP48-3T (EF197994)
99 E. sanguinicola SS-1729T (AY321376)
E. devriesei LMG 14595T (AJ891167)
97 E. pseudoavium NCFB 2138T (Y18356)
E. viikkiensis IE3.2T (HQ378515)
80 E. avium NCFB 2369T (Y18274)
81 E. gilvus ATCC BAA-350T (AJDQ01000009)
72 E. malodoratus ATCC 43197T (ASWA01000002)
54 E. raffinosus NCIMB 12901T (Y18296)
93 E. pallens ATCC BAA-351T (AJAQ01000034)
E. hermanniensis LMG 12317T (AY396047)
97 E. canintestini LMG 13590T (AJ888906)
E. dispar ATCC 51266T (AHYR01000005)
66 E. asini ATCC 700915T (ASVU01000001)
82 E. diestrammenae OLR-24T (JQ650245)
E. phoeniculicola ATCC BAA-412T (AJAT01000017)
88 E. rivorum S299T (FN822765)
E. faecalis V583 (AE016830)
E. termitis LMG 8895T (AM039968)
100 69 E. moraviensis ATCC BAA-383T (AJAS01000015)
99 E. quebecensis CCRI-16985T (GU457262)
E. plantarum CCM 7889T (HQ847537)
67 E. ureilyticus CCM 4629T (AJ276352)
60 E. rotai CCM 4630T (AJ276353)
54 E. ureasiticus CCRI-16986T (GU457264)
E. silesiacus LMG 23085T (AM039966)
E. caccae ATCC BAA-1240T (AJAU01000001)
100 E. casseliflavus ATCC 49996T (AJAM01000006)
E. gallinarum CECT 970T (AJ420805)
99 E. alcedinis L34T (JX948102)
96 E. eurekensis PC4BT (AF445305)
E. lemanii PC32T (AF445301)
E. aquimarinus LMG 16607T (AJ877015)
E. columbae DSM 7374T (AHYW01000008)
100 E. cecorum DSM 20682T (ASWI01000003)
68 93 E. camelliae FP15-1T (EF154454)
66 E. italicus DSM 15952T (AEPV01000109)
E. sulfureus ATCC 49903T (ASWO01000001)
100 E. olivae IGG13.11 (KJ566121)
E. olivae IGG16.11T (JQ283454)
70 E. saccharolyticus subsp. taiwanensis 812T (AB775175)
90 E. saccharolyticus subsp. saccharolyticus ATCC 43076T (ASWN01000003)

Fig. 1. Phylogenetic relationships based on comparison of 16S rRNA gene sequences (1406 nt) from strain IGG16.11^T with those from species of the genus *Enterococcus* described to date. For clarity, only isolates IGG16.11^T and IGG13.11 are shown in the tree. Nucleotide accession numbers of the sequences are given in parentheses. The tree was reconstructed with the neighbour-joining method. Numbers at branches indicate bootstrap values calculated for 1000 subsets for branch-points supported by values >50 %. Bar, 0.005 changes per nucleotide position.

On the basis of phenotypic, genotypic and phylogenetic characteristics, we suggest that the strains described here represent a novel species of the genus *Enterococcus*.

Description of *Enterococcus olivae* sp. nov.

Enterococcus olivae (o.li′vae. L. gen. n. *olivae* of an olive, referring to the isolation of the strains from olive fermentations).

Cells are Gram-stain-positive, non-motile, non-spore forming, egg shaped, 0.5–1 μm in diameter and arranged in pairs or in short chains. They are facultatively anaerobic, but grow better under anaerobic conditions on BHI plus 0.05 % (w/v) L-cysteine agar plates (BHI-Cys). Colonies grow on BHI-Cys after 2 days of incubation. Colonies are about 2–3 mm in diameter, circular, white, flat, with a smooth surface and entire edge, and exhibit the production of a 'ropy'-type exopolysaccharide. Colonies on Glucose Yeast-Extract Peptone medium (GYP) agar plates are circular, low-convex with entire margins, transparent and non-pigmented. Small, red colonies appear on Slanetz–Bartley agar. Positive for hydrolysis of aesculin and starch. Negative for catalase, hydrolysis of gelatin and production of gas from glucose and gluconate. Uses glucose fermentatively. No acidification, coagulation, reduction or liquefaction in skimmed milk after 7 days of growth. Growth in BHI at pH 5.0–9.6, with 0.5–6.5 % NaCl and at 10, 30 and 37 °C, but not at 45 °C. Acid is produced from D-glucose, D-fructose, D-galactose, D-mannose, maltose, aesculin, mannitol, *N*-acetylglucosamine, trehalose, sucrose, D-sorbitol, starch, melibiose, L-sorbose and raffinose. Weakly positive reaction in turanose and D-arabitol. Acid is not produced from glycerol, erythritol, D-/L-arabinose, D-ribose, D-/L-xylose, adonitol, methyl β-xyloside, L-rhamnose, dulcitol, inositol, D-amygdalin, inulin, melezitose, glycogen, xylitol, D-lyxose, D-/L-fucose, L-arabitol, gluconate or 2,5-diketogluconate.

The type strain, IGG16.11^T (=CECT 8063^T=DSM 25431^T), was isolated from Spanish-style green-olive fermentations. The DNA G+C content of the type strain is 40.4 mol%.

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Table 1. Biochemical characteristics that differentiate *E. olivae* sp. nov. from other related enterococci

Strains: 1, *E. olivae* sp. nov. IGG16.11^T; 2, E. camelliae FP15-1^T; 3, *E. italicus* DSM 15952^T; 4, *E. sulfureus* DSM 6905^T; 5, *E. saccharolyticus* subsp. *saccharolyticus* CECT 4309^T; 6, *E. saccharolyticus* subsp. *taiwanensis* 812^T. The biochemical characteristics of *E. saccharolyticus* subsp. *saccharolyticus* CECT 4309^T were experimentally determined in this study. Data for reference type strains were taken from Sukontasing *et al.* (2007), Fortina *et al.* (2004), Martinez-Murcia & Collins (1991), Farrow *et al.* (1984) and Chen *et al.* (2013). +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6
Pigment production	−	−	−	+	−	−
Growth in 6.5 % NaCl	+	−	−	+	+	+
Growth at 10 °C	+	−	W	+	+	−
Production of acid from:						
D-Amygdalin	−	−	−	+	+	+
D-Arabitol	W	−	−	−	+	+
D-Galactose	+	−	+	+	+	W
Lactose	−	−	+	+	+	+
Melezitose	−	−	−	+	+	−
Melibiose	+	−	−	+	+	+
Raffinose	+	−	−	+	+	+
D-Ribose	−	−	−	+	+	−
D-Sorbitol	+	−	+	−	+	+
Turanose	W	−	−	ND	+	+
Potassium 2-ketogluconate	−	−	−	+	+	−
Starch	+	−	+	ND	+	−
DNA G+C content (mol%)	40.4	37.8	39.9–41.1	38.4	37.2	35.7

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