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 812T and from E. saccharolyticus subsp. saccharolyticus ATCC 43076T, respectively. The sequence of the rpoA gene in the isolates was 95 % similar to that of E. saccharolyticus CECT 4309T (=ATCC 43076T). 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 IGG16.11T (=CECT 8063T =DSM 25431T).

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 mammals 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, southwestern 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 1−1 (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 % H2, 10 % CO2 and 80 % N2. 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 IGG16.11T and IGG13.11 are JQ283454 and KJ566121, respectively. The accession numbers for the rpoA gene sequences of E. olivae strains IGG16.11T and IGG13.11 are JQ283455 and KJ566122, respectively.

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

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fermentation yards under study. The isolates were geno-
typed 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’-
TACGgyTACTGTTACGACTT-3’) (Lane, 1991), and 
both DNA strands were sequenced using the primers 7for, 
785F (5’-GGATTAGATACCCBRGTAGTC-3’) and mb16 (5’-
GGCTGCTGCGACGTAAGTAGTAGT-3’) (Kullen et al., 2000) 
and 1510r. In addition, partial amplification and sequen-
cing of 800 bp of the gene rpoA with primers rpoA-21-F 
(5’-ATGATYGARTTGGAAAACC-3’) and rpoA-23-R 
(5’-ACHGTRTRATDCCGCGRCG-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.ezbiolcloud.net; Kim 
et al., 2012), containing type strains with updated, validly 
published prokaryotic names. The global alignment algo-

rithm (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 IGG16.11T and Enterococcus 
saccharolyticus CECT 4309T was determined with a fluoros-
metric 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 
(ΔTm) via fluorescence values. In addition, the G+C 
content of genomic DNA of strain IGG16.11T 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 
812T and of 98.5 % with that of E. saccharolyticus subsp. 
saccharolyticus ATCC 43076T. Similarly, BLASTN 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 4309T (ATCC 43076T). 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 
IGG16.11T and IGG13.11 formed a separate branch within the 
Enterococcus gallinarum group, with E. saccharolyticus 
ATCC 43076T being the most closely related species (Fig. 
1). Bootstrap resampling values of 70 % indicated statist-
cally significant support for the Enterococcus olivae sp. nov. 
IGG16.11T and IGG13.11 and E. saccharolyticus branch. 
The phylogenetic analysis of the rpoA gene sequence showed 
a clustering of E. olivae sp. nov. IGG16.11T 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 IGG16.11T and from E. saccharolyticus 
CECT 4309T was 7.5 °C. This value was above the 5 °C 
ΔTm 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 
IGG16.11T represents a novel species clearly differentiated 
from E. saccharolyticus. The G+C content of genomic 
DNA of strain IGG16.11T was 40.4 mol% (SD, 0.1).

Substrate utilization, the fermentation/oxidation profile, 
acid production and some other physiological character-
istics 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 Svec et al. (2001) and Devriese et 
(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. IGG16.11T 
from their closest phylogenetic relatives, E. italicus, E. 
camelliae, E. saccharolyticus and Enterococcus sulfurueus.
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, ascorbic, mannanot, N-acetylglucosamine, trehalose, sucrose, D-sorbitol, starch, melibiose, D-sorbos and raffinose. Weakly positive reaction in turanose and D-arabitol. Acid is not produced from glycerol, erythritol, D-/L-arabinose, D-ribose, D-/L-xylene, adonitol, methyl β-xylolide, 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, IGG16.11T (≈ CECT 8063T = DSM 25431T), 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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