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

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Three isolates originating from Spanish-style green-olive fermentations in a manufacturing company in the province of Seville, Spain, were taxonomically characterized by a polyphasic approach. This included a phylogenetic analysis based on 16S rRNA gene sequences and multi-locus sequence analysis (MLSA) based on pyrH, recA, rpoA, gyrB and mreB genes. The isolates shared 98.0 % 16S rRNA gene sequence similarity with Vibrio xiamenensis G21^T. Phylogenetic analysis based on 16S rRNA gene sequences using the neighbour-joining and maximum-likelihood methods showed that the isolates fell within the genus Vibrio and formed an independent branch close to V. xiamenensis G21^T. The maximum-parsimony method grouped the isolates to *V. xiamenensis* G21^T but forming two clearly separated branches. Phylogenetic trees based on individual pyrH, recA, rpoA, gyrB and mreB gene sequences revealed that strain IGJ1.11^T formed a clade alone or with *V. xiamenensis* G21^T. Sequence similarities of the *pyrH*, recA, rpoA, gyrB and mreB genes between strain IGJ1.11^T and V. xiamenensis G21^T were 86.7, 85.7, 97.3, 87.6 and 84.8 %, respectively. MLSA of concatenated sequences showed that strain IGJ1.11^T and V. xiamenensis G21^T are two clearly separated species that form a clade, which we named Clade Xiamenensis, that presented 89.7 % concatenated gene sequence similarity, i.e. less than 92 %. The major cellular fatty acids (>5 %) of strain IGJ1.11^T were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega_{6c}$). Enzymic activity profiles, sugar fermentation patterns and DNA G+C content (52.9 mol%) differentiated the novel strains from the closest related members of the genus Vibrio. The name Vibrio olivae sp. nov. is proposed for the novel species. The type strain is IGJ1.11^T (=CECT 8064^T=DSM 25438^T).

At the time of writing, the genus *Vibrio* consisted of 111 species according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN; http://www.bacterio.net).

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Abbreviations: LAB, lactic acid bacteria; ML, maximum-likelihood; MLSA, multi-locus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* gene sequences of strain IGJ1.11^T are JQ283456, JQ283457, JQ283458, JQ283459, KP162090 and KP162091, respectively.

Three supplementary tables and nine supplementary figures are available with the online Supplementary Material.

Recently, species of the genus *Vibrio* have been grouped in 19 clades based on multi-locus sequence analysis (MLSA) using eight housekeeping genes (Sawabe *et al.*, 2013). Vibrios are Gram-negative, usually motile rods, halophilic, mesophilic and chemo-organotrophic, and have a facultatively fermentative metabolism (Baumann *et al.*, 1984). Species from this genus have been commonly isolated from aquatic environments, usually from marine environments, both as free-living bacteria and as symbionts or parasites of fish, molluscs and crustaceans (Thompson *et al.*, 2004).

Spanish-style green-olive fermentations are characterized by an ecological succession of diverse microbial species. Their preparation is carried out by an initial alkali treatment [1.8-3.5% (w/v) NaOH] of the green fruits, in order to remove bitterness and allow the subsequent growth of lactic acid bacteria (LAB) through the neutralization and

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washing of inhibitory phenolic compounds (Rejano et al., 2010). Once the alkali is removed, fruits are washed once or twice with water and finally covered with brine [10-12 % (w/v) NaCl]. In this brine, a spontaneous fermentation takes place in which at least three different stages have been identified (Fernandez et al., 1995). During the first stage, usually lasting 3-10 days, fermentation is conducted by the indigenous alkali-tolerant microbiota that contaminates the fruits as well as the environment (de Castro et al., 2002). This microbiota is responsible for lowering the initial high pH (10-11) to values close to 6-7, more appropriate for the growth of LAB, which are also present as contaminants (Sanchez et al., 2001). Later, the usual alkali-tolerant microbiota disappear as a consequence of the lowering of the pH due to the growth of LAB, mainly strains of Lactobacillus pentosus, which is characteristic of the second fermentation stage (de Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba & Jiménez-Diaz, 2012). In the final stage of the fermentation, sugars are exhausted and LAB population declines steadily, thus starting the storage period.

Recently, we have carried out an exhaustive analysis of the microbiota associated to Spanish-style green olive fermentations, attending to its dynamics along the time (Lucena-Padrós *et al.*, 2014). During this study, we found nine isolates at the initial stage of the fermentation whose partial 16S rRNA gene sequences showed similarity ($\leq 97\%$) to the species *Vibrio furnissii* and *Vibrio fluvialis*, suggesting that they could constitute a novel species of the genus *Vibrio*. As stated above, this first stage of the fermentation process is characterized by high pH and salt values, which is an ideal environment for the grown of alcaliphilic and halophilic bacteria such as species of the genus *Vibrio*.

The nine isolates were recovered from brine samples from six different 10-tonne fermenters at the first two weeks of the fermentation process as described previously (Lucena-Padrós *et al.*, 2014). Briefly, samples were serially diluted and spread onto plates of brain heart infusion (BHI; Biokar Diagnostics) supplemented with 0.05% (w/v) L-cysteine (AppliChem). Plates were incubated anaerobically at 30 °C for 48 h in a DG250 Anaerobic Workstation (Don Whitley Scientific), with a gas mixture consisting of 10% H₂/10% CO₂/80% N₂.

The nine isolates, were genotyped by random amplified polymorphic DNA (RAPD) analysis using the primer ISS1rev (5'-GGATCCAAGACAACGTTTCAAA-3') (Veyrat *et al.*, 1999), following the protocol of Maldonado-Barragan *et al.* (2013). Three representative isolates (IGJ1.11^T, IGJ8.11 and IGJ10.11) showing a different RAPD profile were selected for further characterization (Fig. S1, available in the online Supplementary Material).

DNA from pure cultures was isolated according to the protocol of Lazo *et al.* (1987). A fragment of the 16S rRNA gene was amplified with the primer pair 7 for (5'-AGAGTTTGATYMTGGCTCAG-3') and 1510r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). The

rpoA, recA, gyrB, pyrH and mreB genes were amplified for MLSA according to Thompson et al. (2005) and Sawabe et al. (2007). The identification of the isolates and their phylogenetic neighbours was carried out by the BLASTN program on the basis of 16S rRNA gene sequence data obtained (Altschul et al., 1997) against the database containing type strains with updated validly published prokaryotic names, by using the EzTaxon-e server (http://eztaxon-e. ezbiocloud.net/; Kim et al., 2012). The calculation of pairwise sequence similarity was done using the global alignment algorithm (Myers & Miller, 1988), which was implemented at the EzTaxon-e server. The pyrH, recA, rpoA, gyrB and mreB nucleotide sequences similarities were determined using the MEGALIGN software in the DNASTAR package. The DNA sequences belonging to type strains of species of the genus Vibrio were obtained from the GenBank database.

To test the evolutionary relationships of the genus Vibrio, phylogenetic analysis based on individual and concatenated sequences was performed. For this, the sequences were aligned by using the CLUSTAL W method (Thompson et al., 1994) with the MEGA 5 (version 5.2) software (Tamura et al., 2011). To reconstruct the phylogenetic trees based on individual and concatenated pyrH, recA, rpoA, gyrB and mreB genes, we used regions 172-577, 440-937, 86-875, 441-1035 and 386-923 [V. cholerae O1 Eltor N16961 (Gen-Bank accession no. AE003852) numbering], respectively. These regions were in the range of those used by Sawabe et al. (2007, 2013). The phylogenetic trees were reconstructed based on the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximumlikelihood (ML) (Felsenstein, 1981) methods. For NJ and ML analysis, distance matrices were calculated using Kimura's two-parameter correction. ML analysis was carried out using a heuristic search option (Nearest-Neighbour-Interchange; NNI). MP analysis was performed using the Subtree-Pruning-Regrafting (SPR) search method. Bootstrapping analysis (1000 replicates) was done to study the stability of the groupings. The GenBank accession numbers for genes used in the MLSA are given in Table S1.

The degree of DNA–DNA relatedness between strain IGJ1.11^T and its closest phylogenetic neighbour, *V. xiamenensis* DSM 22851^T (=G21^T) was determined by the fluorimetric method as described by Gonzalez & Saiz-Jimenez (2005). This method measures the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m) using a realtime PCR thermocycler that obtains fluorescence determinations. The G+C content of genomic DNA of strain IGJ1.11^T was also determined by the fluorimetric method described by Gonzalez & Saiz-Jimenez (2002). The results of both DNA–DNA relatedness and DNA G+C content were expressed as mean percentage values \pm sD, based on eight and three independent experiments, respectively.

Substrate utilization, the sugar fermentation/oxidation profile, acid production and other biochemical characteristics were examined at the Spanish type Culture Collection

(CECT; Universitat de València, Paterna, Valencia, Spain) using the API 20 E fermentation kit (bioMérieux) after incubation for 24 h at 30 °C, according to the manufacturer's instructions. Cell morphology, size and motility were examined at the CECT using a phase-contrast Leica DMRB microscope. Cells of a 14 h-old culture grown at 30 °C on marine agar (MA) were examined. Micrographs were taken using a Leica EC3 digital camera and processed using the software Leica LAS EZ (Leica Microsystems). Cell size was estimated from a digital image calculating mean (n = 10) length and width. The temperature range for growth was determined by culturing the isolate in MA at 4-45 °C. Growth at pH 4.0-10.0 was tested at 30 °C in BHI-cys with the pH adjusted with HCl (for pH 4.0-7.0) or NaOH (for pH 7.0-10.0). Sensitivity of growth to NaCl concentration was determined with modified BHI-cys, in which the concentration of NaCl ranged from 1 to 10% (w/v). Growth under anaerobic conditions was determined by incubation on BHI-cys as described above. Growth on thiosulfate-citrate-bile-sucrose (TCBS; Difco) plates was determined at 30 °C. Catalase activity was determined by bubble production in a 10 % (v/v) H_2O_2 solution.

Analysis of whole-cell fatty acids was carried out at CECT following the protocol recommended by the MIDI Microbial Identification System (Sasser, 1990) using a culture grown on MA for 14 h at 30 °C. Analysis of fatty acid

methyl esters was carried out with an Agilent Technologies 6850 gas chromatograph with the Sherlock 6.1 database using the Sherlock Microbial Identification System (MIDI).

The 16S rRNA gene sequences of isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 were 100% identical. The BLASTN analysis of these 16S rRNA gene sequences (1437 bp) showed that isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 belong to the genus Vibrio. The type strains of the most closely related species of the genus Vibrio were V. xiamenensis G21^T, V. furnissii CIP 102972^T, V. fluvialis NCTC 11327^T, V. variabilis R-40492^T and Vibrio hepatarius LMG 20362^T, which showed 98.0, 97.6, 97.4, 97.2 and 97.1 % 16S rRNA gene sequence similarity, respectively (Table S2). Recently, Kim et al. (2014) have proposed 98.65 % 16S rRNA gene sequence similarity as the threshold for differentiating two species, being in the threshold range (98.2-99.0%) previously suggested by other studies (Meier-Kolthoff et al., 2013; Stackebrandt & Ebers, 2006). The 16S rRNA gene sequence similarity between isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 and V. xiamenensis G21^T (98.0%) is below 98.65%, thus indicating the isolates represent a distinct species.

The phylogenetic analysis inferred from the 16S rRNA sequences, using the NJ and ML methods, showed that the isolate $IGJ1.11^{T}$ forms an independent branch close to *V. xiamenensis* $G21^{T}$ (Fig. 1 and Fig. S2). The MP method grouped isolate $IGJ1.11^{T}$ to *V. xiamenensis* $G21^{T}$;



Fig. 1. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship of strain IGJ1.11^T to some members of the genus *Vibrio*. The tree was reconstructed using the neighbour-joining method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points >50%. *Photobacterium damselae* subsp. *damselae* CIP 102761^T was used as an outgroup. GenBank sequence accession numbers are given in parentheses. Bar, 0.005 nt changes per nucleotide position.

however, bootstrap resampling values (64 %) support that isolate $IGJ1.11^{T}$ and *V. xiamenensis* $G21^{T}$ form two clearly separated branches (Fig. S3).

Nowadays, MLSA is considered a reliable technique to reconstruct the phylogeny of the genus *Vibrio* (Thompson *et al.*, 2005; Sawabe *et al.*, 2007, 2013). The *pyrH*, *recA*, *rpoA*, *gyrB and mreB* gene sequences of the isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 were 100 % identical. Phylogenetic trees based on *pyrH*, *recA*, *rpoA*, *gyrB and mreB* gene sequences revealed that strain IGJ1.11^T formed a clade alone or with *V. xiamenensis* (Figs S4–S8). Sequence similarities of the *pyrH*, *recA*, *rpoA*, *gyrB and mreB* genes between strain IGJ1.11^T and *V. xiamenensis* G21^T were 86.7, 85.7, 97.3, 87.6, and 84.8 %, respectively (Table S2). These values are below the threshold values of 98 %, 94 % and 94 % for *rpoA*, *recA* and *pyrH*, respectively, recommended for delineating species in the genus *Vibrio* (Thompson *et al.*, 2005; Sawabe *et al.*, 2007).

The phylogenetic tree based on concatenated sequences *pyrH, recA, rpoA, gyrB and mreB*, showed that isolate

IGJ1.11^T forms a clade with *V. xiamenensis* G21^T (Fig. 2). Bootstrap resampling values (100%) indicated that the novel isolates and *V. xiamenensis* are two clearly separated species. Interestingly, all species of the genus *Vibrio* used in this study were grouped into their corresponding clade according to the recent update of the *Vibrio* clades carried out by Sawabe *et al.* (2013) (Fig. 2). In the light of the phylogenetic analysis based on MLSA of five concatenated sequences (*pyrH*, *recA*, *rpoA*, *gyrB and mreB*), we propose that strainIGJ1.11^T represents a novel species which, together with *V. xiamenensis* G21^T, could form a new clade, i.e. the clade *Xiamenensis* (Fig. 2).

Thompson *et al.* (2005) suggested that species of the genus *Vibrio* are defined as a group of strains that share more than 95% similarity among MLSA datasets. Recently, Sawabe *et al.* (2013) showed that <98% of 8-gene-concatenated nucleotide sequence similarity may allow us to define a species boundary. Thus, strains below this threshold value would qualify as members of separate species. Strain IGJ1.11^T showed an 89.7% gene-concatenated nucleotide sequence similarity to



Fig. 2. Phylogenetic tree based on concatenated sequences of the *recA* (498 bp), *rpoA* (790 bp), *pyrH* (406 bp), *gyrB* (595 bp) and *mreB* (538 bp) genes, using the neighbour-joining method. Bootstrap values were expressed based on 1000 replications; only values \geq 50% are shown. Clades to which the different species belong are indicated. *Photobacterium damse-lae* subsp. *damselae* ATCC 33539^T was used as an outgroup. Bar, 2% estimated sequence divergence.

its nearest neighbour, *V. xiamenensis* G21^T, thus indicating it represents a novel species of the genus *Vibrio* (Table S2).

The difference in melting temperature between genomic DNA from strain IGJ1.11^T and that from *V. xiamenensis* DSM 22851^T (=G21^T) was 6.4 °C (sD=1.0; n = 8). This value was above the 5 °C ΔT_m recommended as cut-off point for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA relatedness results confirmed that strain IGJ1.11^T represents a novel species clearly differentiated from *V. xiamenensis*. The G+C content of genomic DNA of strain IGJ1.11^T was 52.9 mol% (sD=0.95, n = 3), which was within the range of the genus *Vibrio*.

The three analysed isolates (IGJ1.11^T, IGJ8.11 and IGJ10.11) were shown to possess the same biochemical profile

Table 1. Biochemical and metabolic characteristics that differentiate strain IGJ1.11^T from related species of the genus *Vibrio*

Strains: 1, IGJ1.11^T; 2, *V. xiamenensis* G21^T; 3, *V. furnissii* CAIM 518^T; 4, *V. fluvialis* CAIM 593^T; 5, *V. proteolyticus* NBRC 13287^T. Data for reference type strains were taken from Brenner *et al.* (1983), Gao *et al.* (2012), Farmer & Hickman-Brenner (2006), Farmer *et al.* (2005), Kalina *et al.* (1984) and Noguerola & Blanch (2008). Biochemical characteristics of strain IGJ1.11^T were determined using the API 20E identification system (bioMérieux). Growth of strain IGJ1.11^T on TCBS plates was determined at 30 °C, while growth in 10% NaCl was determined in BHI-cys at 30 °C. The G+C content of genomic DNA of strain IGJ1.11^T was determined by the fluorimetric method of Gonzalez & Saiz-Jimenez (2002). All strains were negative for H₂S production. +, Positive; –, negative; v, variable; ND, no data available. ^y, yellow colonies.

Characteristic	1	2	3	4	5
β-Galactosidase	+	+	_	+	_
Arginine dihydrolase	_	_	+	+	+
Lysine decarboxylase	_	_	_	_	+
Citrate utilization	+	-	+	+	+
Urease	-	-	-	_	V
Indole production	_	_	_	_	+
Voges–Proskauer	_	_	_	_	+
Gelatinase	+	-	+	+	+
Catalase	-	+	ND	ND	-
Acid production from:					
D-Sorbitol	-	-	-	_	+
L-Rhamnose	+	+	+	_	-
D-Sucrose	+	+	+	+	-
D-Melibiose	+	-	_	-	_
Amygdalin	+	+	ND	ND	-
L-Arabinose	+	+	+	+	-
Growth in/on:					
10% (w/v) NaCl	+	+	-	-	+
TCBS medium	$+^{\gamma}$	_	$+^{\gamma}$	$+^{y}$	ND
DNA G+C content (mol%)	52.9	46.0	50.4	49.3–50.3	50.5

IGJ8.11 and IGJ 10.11 were negative. Table 1 shows phenotypical tests that are useful for the differentiation of strain IGJ1.11^T from the closest phylogenetic relatives, i.e. *V. xiamenensis*, *V. fluvialis*, *V. furnissii* and *V. proteolyticus*. Strain IGJ1.11^T (and strains IGJ8.11 and IGJ10.11) could be easily differentiated from its neighbours by the ability to produce acid from melibiose. Growth at different temperatures, pH and NaCl concen-

Growth at different temperatures, pH and NaCl concentrations and other phenotypical features are given in the species description. The major cellular fatty acids (>5%) of strain IGJ1.11^T were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Although similar to other species of the genus *Vibrio*, strain IGJ1.11^T showed a fatty acid profile different from those of its phylogenetically closest species (Table S3).

described in Table 1 for the type strain, except for citrate utilization, where strain $IGJ1.11^{T}$ was positive but strains

In conclusion, on the basis of phenotypic, DNA–DNA reassociation, genotypic and phylogenetic characteristics, we suggest that the strains studied represent a novel species of the genus *Vibrio*, for which we propose the name *Vibrio* olivae sp. nov. (type strain IGJ1.11^T).

Description of Vibrio olivae sp. nov

Vibrio 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-negative, lightly curved rods $(1.4 \ \mu m \times 0.6 \ \mu m; Fig S9)$ and non-motile. Colonies on BHI are circular, convex, with a uniform edge, mucoid and white to cream with a diameter of 2-3 mm after incubation at 37 °C for 24 h. Colonies on TCBS are circular, convex, with a uniform edge, mucoid and yellow with a diameter of 4 mm after incubation at 37 °C for 24 h. Grows in BHI broth (this medium typically contains 0.5% NaCl), not requiring added NaCl for its growth, at pH 5.0-9.0, and in 1-10.0% NaCl. Growth occurs at 15-42 °C, but not at 10 °C or below, nor at 45 °C or above. Optimal growth is achieved aerobically at 30-37 °C, at pH 7.4 in BHI broth medium. Grows anaerobically in BHI-cys medium at 30 °C, but requires at least 1% NaCl for growth. Acid is produced from D-glucose, D-mannitol, sucrose, melibiose, amygdalin, L-arabinose and Lrhamnose, but not from inositol or D-sorbitol. Positive for β -galactosidase and oxidase activities, but negative for catalase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, H₂S production, indole production, and Voges-Proskauer reaction. Positive for hydrolysis of gelatin. Citrate utilization is straindependent (IGJ1.11^T is positive, but strains IGJ8.11 and IGJ 10.11 are negative). The predominant cellular fatty acids (>5%) are summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$).

The type strain, $IGJ1.11^{T}$ (=CECT 8064^T = DSM 25438^T), was isolated from Spanish-style green olive fermentations. The DNA G+C content of the type strain is 52.9 mol%.

Two additional strains of the species are IGJ8.11 and IGJ 10.11, also isolated from green olive fermentations.

Acknowledgements

This research was funded by the Spanish Ministry of Economy and Competitiveness (MINECO) and by the Junta de Andalucía Excellence Projects, through projects AGL2012-33400 and AGR-07345, respectively. These projects included FEDER funds. A. M. B. is a Doctor hired by the Junta de Andalucía Excellence Project AGR-07345. H. L. P. was the recipient of a grant awarded by the MINECO as part of the Project AGL2012-33400. We want to express our gratitude to Mari Carmen Macián Rovira, from CECT, for her skilful assistance and helpful advice.

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