Virgibacillus olivae sp. nov., isolated from waste wash-water from processing of Spanish-style green olives

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Four bacterial strains (E₃₀8^T, E₅₅49, I₃₀77 and N₃₀129) were isolated from the residual wash-water produced during the processing of Spanish-style green table olives. The isolates were subjected to a polyphasic taxonomic study using phenotypic, phylogenetic and genotypic methods. The bacteria were Gram-positive, spore-forming rods. Moreover, they were heterotrophs that were able to utilize cellobiose, glucose, mannose and rhamnose as carbon sources. The G + C content of their genomic DNA ranged from 30.7 to 33.4 mol%. The major cellular fatty acids found in strain E₃₀8^T were iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:0} and anteiso-C_{17:0}. DNA–DNA hybridization shows 76.2–88.3 % relatedness among the four strains. The 16S rRNA gene sequence of isolate E₃₀8^T shows that it belongs to the genus *Virgibacillus*, with the highest sequence similarity (99 %) to *Virgibacillus marismortui* 123^T. However, phenotypic differences and DNA–DNA relatedness between strain E₃₀8^T and *V. marismortui* ATCC 700626^T of less than 47 % suggest the placement of these strains within a novel species of the genus *Virgibacillus*. The name *Virgibacillus olivae* sp. nov. is proposed, with strain E₃₀8^T (=LMG 23503^T = DSM 18098^T) as the type strain.

During the preparation of edible olives according to the procedure used to give so-called ‘Spanish green olives’, residual wash-water (EWW, edible-olive wash-water) is generated as a waste product. In this process, the olives are treated with brines which contain 10–13 % (w/v) NaCl, in which they undergo lactic fermentation (Brenes & de Castro, 1997). The brines represent a large proportion of the total contaminating discharges from the olive industry. However, specific treatments for these wash-waters are either non-existent or ineffective (Romero Barranco *et al.*, 2001). To obtain a better insight into the microbial community present in EWW, we analysed the microbial content. In the present study, we report the description of a novel aerobic, mesophilic, endospore-forming organism isolated from EWW that was able to grow in 0–20 % NaCl.

Initially, 180 strains were isolated from samples of EWW (provided by AgroSevilla S.A., La Roda, Sevilla, Spain) obtained at different stages of the edible olive processing. Isolation was performed by the standard dilution-plating technique on HM medium (Ventosa *et al.*, 1982)

supplemented with a balanced mixture of sea salts with 10 % (w/v) NaCl (Rodríguez-Valera *et al.*, 1981) at 30 °C. Strains were maintained on HM medium (Ventosa *et al.*, 1982) supplemented with 5 % (w/v) sea-salt solution (referred to here as HM 5 % sea salts medium) (Rodríguez-Valera *et al.*, 1981). The predominant strains present in this wash-water were Gram-positive, endospore-forming bacilli. For this study, of the 180 isolated strains, four were selected on the basis of their similar physiological characteristics.

Cell morphology was examined by light microscopy and transmission electron microscopy using cells from exponentially growing cultures. Endospores were stained according to the Schaeffer–Fulton method (Smibert & Krieg, 1981) from a 10-day culture on HM 5 % sea salts medium. Growth at different temperatures (10, 20, 25, 30, 45, 55, 60 and 70 °C) and pH (2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) was determined on HM 5 % sea salts medium. Growth at different salt contents (0, 3, 5, 10, 15, 20, 25 and 30 %, w/v) was investigated on HM medium. Catalase activity was determined by bubble production in a 10 % (v/v) H₂O₂ solution. Oxidase activity was determined on filter paper moistened with a 1 % (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Aerobic nitrate and nitrite reduction, methyl red, indole production and Voges–Proskauer tests and hydrolysis of starch, casein, aesculin, gelatin, tyrosine and Tweens 20, 40 and 80 were all assayed according to Claus & Berkeley (1986). Utilization of various

Abbreviation: EWW, edible-olive wash-water.

The GenBank/EMBL/DBJ accession number for the 16S rRNA sequence of strain E₃₀8^T is DQ139839.

A 16S rRNA gene sequence-based maximum-parsimony tree is available as supplementary material in IJSEM Online.

substrates as sole carbon and energy sources and acid production from carbohydrates were determined as described previously (Ventosa *et al.*, 1982). Carbohydrates were added at 0.2 % (w/v), organic acids at 0.1 % (w/v) and alcohols at 0.1 % (v/v). Strains were incubated at 30 °C. All assays were performed in duplicate. Antimicrobial susceptibility was tested in HM 5 % sea salts medium according to the method described by Bauer *et al.* (1966).

Cellular fatty acid analysis was performed from strains grown on liquid HM 5 % sea salts medium for 24 h at 30 °C. Quantitative analysis of cellular fatty acid composition was performed by high-resolution GLC by the DSMZ Identification Service (Braunschweig, Germany).

DNA was purified using the method of Lind & Ursing (1986). The G + C content of genomic DNA was calculated from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) obtained at 260 nm with a Perkin-Elmer UV-Vis Lambda3B spectrophotometer fitted with a temperature program accessory. The T_m was determined by the graphical method described by Ferragut & Leclerc (1976) and the G + C content was estimated from this temperature using the equation of Owen & Hill (1979).

The 16S rRNA gene was amplified by PCR using standard protocols with primers 16F27 (forward; 5'-AGAGTTTGA-TCMTGGCTCAG-3') and 16R1525 (reverse; 5'-AAGGAG-GTGWTCCARCC-3') (Pharmacia Biotech). PCR products were purified using Microcon-100 concentrators (Amicon) and sequenced directly using an Applied Biosystems 373A DNA sequencer. *Taq* cycle sequencing was performed with fluorescent dye-labelled dideoxynucleotides (Perkin-Elmer) and the following oligonucleotides: 16F357 (5'-CTCCTA-CGGGAGGCAGCAG-3'), 16R519 (5'-GWATTACCGCG-GCKGCTG-3') and 16F945 (5'-GGGCCCCGACAAGCG-GTGG-3').

The sequences obtained were compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases. A phylogeny analysis was made using MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL W (Chenna *et al.*, 2003). Distances and clustering were determined using neighbour-joining and maximum-parsimony algorithms. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications). DNA–DNA hybridization was conducted following the method of Lind & Ursing (1986) with the modifications described by Ziemke *et al.* (1998).

Phenotypic characteristics common to all four strains are given in the species description. Table 1 shows differential phenotypic characteristics between the four isolates and Table 2 shows the characteristics that distinguish strain E₃₀8^T from other related *Virgibacillus* species. The major fatty acids (greater than 1 %) found in strain E₃₀8^T were iso-C_{14:0} (2.05 %), C_{14:0} (1.25 %), iso-C_{15:0} (33.71 %), anteiso-C_{15:0} (28.43 %), C_{15:0} (1.12 %), iso-C_{16:0} (3.93 %), C_{16:1}ω7c alcohol (1.33 %), C_{16:1}ω11c (1.69 %), C_{16:0}

Table 1. Differential phenotypic characteristics between isolates E₃₀8^T, E₅₅49, I₃₀77 and N₃₀129

Characteristic	E ₃₀ 8 ^T	E ₅₅ 49	I ₃₀ 77	N ₃₀ 129
Growth at/in:				
10 °C	–	+	–	–
55 °C	–	+	–	–
pH 9	–	+	–	+
15 % salts	+	+	–	–
20 % salts	+	–	–	–
Growth on:				
Sucrose	–	+	+	–
D-Xylose	–	+	–	–
L-Alanine	–	–	–	+
L-Lysine	–	+	–	–
Fumarate	+	–	+	–
DL-Malate	–	–	+	+
Propionate	+	–	+	+
Hydrolysis of:				
Aesculin	+	+	+	–
Casein	+	–	–	–

(3.07 %), iso-C_{17:0} (10.13 %), anteiso-C_{17:0} (9.21 %), C_{18:1}ω9c (1.15 %) and C_{18:0} (1.24 %).

The DNA G + C contents for strains E₃₀8^T, E₅₅49, I₃₀77 and N₃₀129 were respectively 33.4, 30.7, 30.8 and 32.4 mol%. Partial 16S rRNA gene sequences (approx. 500 bp) from the four strains showed similarities to the sequence of *Virgibacillus marismortui* 123^T of between 97 and 99 %. The almost complete 16S rRNA gene sequence of strain E₃₀8^T (1564 bp) shared 99 % similarity with *V. marismortui* 123^T and 96 % similarity with the type strains of *Virgibacillus proomii*, *Virgibacillus dokdonensis* and *Virgibacillus panthothenticus*. The phylogenetic tree constructed using the neighbour-joining algorithm showed that strain E₃₀8^T exhibited the closest phylogenetic affinity to the *Virgibacillus* cluster (Fig. 1). The maximum-parsimony algorithm gave a similar result (data available as Supplementary Fig. S1 in IJSEM Online).

The results of DNA–DNA hybridization between strain E₃₀8^T and isolates E₅₅49, I₃₀77, N₃₀129 showed relative binding ratios of 76.2, 88.3 and 78.9 %, respectively. These results showed that the four strains belonged to the same species, because the values were > 70 %. DNA–DNA relatedness between E₃₀8^T and *V. marismortui* ATCC 700626^T was 46.5 %, indicating that strain E₃₀8^T is genotypically distinct from the type strain of this species.

On the basis of the phenotypic, genotypic, chemotaxonomic and phylogenetic data of this study, we conclude that isolates E₃₀8^T, E₅₅49, I₃₀77 and N₃₀129 are different strains that belong to the same species and constitute a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus olivae* sp. nov. is proposed.

Table 2. Characteristics that distinguish between strain E₃₀8^T and other related *Virgibacillus* species

Taxa: 1, strain E₃₀8^T; 2, *V. marismortui*; 3, *V. pantothenticus*; 4, *V. proomii*; 5, *V. salexigens*; 6, *V. carmonensis*; 7, *V. necropolis*; 8, *V. halodenitrificans*; 9, *V. dokdonensis*; 10, *V. koreensis*. Data were obtained from this study and from Arahall *et al.* (1999, 2000), Heyrman *et al.* (2003) and Wainø *et al.* (1999) (*V. marismortui* and *V. salexigens*), Heyndrickx *et al.* (1999) and Heyrman *et al.* (2003) (*V. pantothenticus*), Heyndrickx *et al.* (1999) and Garabito *et al.* (1997) (*V. proomii*), Heyrman *et al.* (2003) (*V. carmonensis* and *V. necropolis*), Yoon *et al.* (2004) (*V. halodenitrificans*), Yoon *et al.* (2005) (*V. dokdonensis*) and Lee *et al.* (2006) (*V. koreensis*). +, Positive; –, negative; v, variable; w, weakly positive; ND, no data available. All species are rod-shaped, motile and positive for catalase.

Characteristic	1	2	3	4	5	6	7	8	9	10
Spore shape*	S, E	E	S, E	S, E	E	S, E	E	E	S, E	E
Spore position†	T, ST	T, ST	T, ST	T, ST	C, ST, T	ST	C, S, ST	T, ST	T, ST	T
Anaerobic growth	–	–	+	+	–	–	–	–	–	+
Growth at/in:										
15 °C	–	+	+	+	+	+	+	+	+	+
50 °C	–	+	+	+	–	–	–	–	+	–
0.5 % NaCl	+	–	w	w	–	–	w	–	+	w
20 % NaCl	+	–	–	–	+	–	–	+	+	–
pH 10	–	–	–	–	+	ND	ND	–	ND	–
Utilization of:										
D-Glucose	+	–	w	w	w	–	+	+	+	ND
Lactose	–	+	–	w	+	–	+	+	ND	ND
D-Fructose	+	–	–	–	–	–	–	+	+	ND
Sucrose	–	+	–	+	+	+	+	+	+	ND
Cellobiose	+	–	w	w	+	w	+	ND	+	ND
D-Mannose	+	–	+	+	+	–	w	+	+	ND
Acid production from:										
D-Glucose	–	+	+	+	w	–	w	+	+	w
<i>myo</i> -Inositol	–	–	–	+	–	–	–	–	+	–
D-Fructose	+	+	–	+	w	–	w	+	+	+
D-Galactose	–	–	–	+	w	–	–	+	+	–
D-Mannose	–	+	–	+	w	–	w	+	+	–
L-Rhamnose	–	–	+	v	–	–	–	–	–	–
Hydrolysis of:										
Starch	+	–	+	ND	–	ND	ND	–	+	ND
Casein	+	+	+	+	+	+	+	+	+	ND
Gelatin	+	+	+	v	+	–	w	+	+	–
Aesculin	+	+	+	+	+	w	–	–	+	+
Nitrate reduction	+	+	v	–	–	+	+	+	–	–
DNA G + C content (mol%)‡	33.4	40.9	37.6	36.9	39.5	38.9	37.3	38	36.7	41

*E, Ellipsoidal; S, spherical.

†C, Central; ST, subterminal; T, terminal.

‡Data for type strains.

Description of *Virgibacillus olivae* sp. nov.

Virgibacillus olivae (o'li.vae. L. gen. n. *olivae* of an olive, referring to the isolation of the first strains from olive processing waste-water).

Cells are straight or slightly curved, rounded-ended, Gram-positive rods (1.8 × 0.26 µm), motile by means of peritrichous flagella, occurring singly or in pairs and occasionally in short chains. Endospore formation is slow; the endospores are nearly spherical or ellipsoidal and appear in a terminal or subterminal position. Colonies on HM 5 % sea

salts medium after 24 h at 30 °C are yellow-cream, semi-translucent, oval with regular margins that may spread along the inoculation streak, slightly transparent at the edge. They have glossy surfaces and are 0.5–2 mm in diameter. Grows at 20–45 °C and pH 4–8. Halotolerant, being capable of growing in sea salts concentrations from 0 to 10 % (w/v). Optimum growth at 30 °C, pH 7 and 5 % (w/v) sea salts. Catalase and oxidase are positive. Nitrate and nitrite are reduced. Hydrolyses starch and gelatin, but not tyrosine or Tween 20, 40 or 80. Citrate is not used as a sole carbon and energy source. Indole, Voges–Proskauer and methyl red tests are negative. No growth on cetrimide agar. Able to use

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequence data showing the phylogenetic position of strain E₃₀8^T, *Virgibacillus* species and other related taxa. GenBank accession numbers used in the phylogenetic analysis are given in parentheses. Only bootstrap values above 50 % are shown (1000 replications). Bar, 0.01 substitutions per nucleotide position.

cellobiose, D-fructose, D-mannose, L-rhamnose and D-glucose as sole carbon and energy sources but not D-galactose or maltose. Acid is produced from D-fructose but not from D-glucose, cellobiose, D-mannose, sucrose, D-galactose, D-xylose, L-rhamnose, D-mannitol, *myo*-inositol or adonitol. L-Cysteine, L-histidine, L-methionine and L-valine are not used as sole sources of carbon, nitrogen and energy. Resistant to kanamycin, amikacin, clindamycin, nalidixic acid, polymyxin B, streptomycin and sulfamides. Sensitive to ampicillin, gentamicin, rifampicin, erythromycin, cephalotin and penicillin. The DNA G+C content is 30.7–33.4 mol% (T_m method).

The type strain is E₃₀8^T (=LMG 23503^T=DSM 18098^T). The type strain and three other strains were isolated from wastewater of Spanish green olive processing. The description of the type strain is the same as that of the species. Additionally, this strain is able to grow in a medium containing 20 % (w/v) sea salts. Aesculin and casein are hydrolysed. Fumarate and propionate can be used as sole carbon and energy sources. Sensitive to tetracycline and resistant to chloramphenicol. The main fatty acids (making up 81 % of the total) are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 33.4 mol% (T_m method).

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