

Vibrio hispanicus sp. nov., isolated from *Artemia* sp. and sea water in Spain

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Three Gram-negative, small, motile, rod-shaped bacteria were isolated from *Artemia* sp. and sea water in Barcelona, Spain, during 1990 and 1991. They were fermentative, oxidase-positive, sensitive to vibriostatic agent O/129, arginine dihydrolase-positive, lysine and ornithine decarboxylase-negative and grew in the absence of NaCl. They differed from phenotypically related species by their ability to grow at 4 °C and utilize L-rhamnose. Cloning of the 16S rRNA gene of the type strain produced two different 16S rRNA gene sequences, which differed by 15 bases (0.99 %); comparison of these sequences with those deposited in GenBank showed close relationships with *Vibrio proteolyticus* (97.6 % similarity), *Vibrio diazotrophicus* (97.9 %), *Vibrio campbellii* (96.8 %) and *Vibrio alginolyticus* (96.8 %), among others. DNA–DNA hybridization levels with the closest phylogenetically related *Vibrio* species were <26.4 %. Sufficient evidence is provided to support the identity of the three strains analysed as members of a novel species of the genus *Vibrio*, for which the name *Vibrio hispanicus* sp. nov. is proposed, with the type strain LMG 13240^T (= CAIM 525^T = VIB 213^T).

Artemia spp. nauplii and cysts have a diverse bacterial composition and many of the genera and species are found commonly in sea water; it has therefore been suggested that the microflora of *Artemia* nauplii is influenced significantly by that of the surrounding water (Igarashi *et al.*, 1989). *Artemia* spp. cysts have been found to contain few bacteria, i.e. less than one bacterium per cyst (Austin & Allen, 1982; Dehasque *et al.*, 1991); after hatching, bacterial density on TCBS agar (which presumably consists of vibrios) can increase dramatically to levels of 10³ c.f.u. per nauplius (Sahul Hameed & Balasubramanian, 2000) or 10⁶–10⁸ c.f.u. (ml *Artemia* nauplii homogenate)^{−1} (López-Torres & Lizárraga-Partida, 2001), and up to 10⁶ c.f.u. ml^{−1} in surrounding water (Austin & Allen, 1982; Dehasque *et al.*, 1991). This suggests that *Vibrio* spp. originate in sea water and then colonize *Artemia* when it hatches, exposing itself for the first time to the surrounding environment.

The taxonomic composition of *Artemia* bacterial cultures

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Abbreviations: FAFLP, fluorescent AFLP; VR, variable region.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of LMG 13240^T clones 1 and 2 are AY254039 and AY254040, respectively.

A graph showing growth of *Vibrio hispanicus* sp. nov. at different NaCl concentrations is available as supplementary material in IJSEM Online.

has been reported to contain a very high proportion of vibrios. In three marine fish hatcheries, 58–87 % of isolates were identified as members of the genus *Vibrio* by fatty acid methyl ester analysis (Verdonck *et al.*, 1994), although the proportion of vibrios in cysts is <2 % (Austin & Allen, 1982; López-Torres & Lizárraga-Partida, 2001) or non-existent (Igarashi *et al.*, 1989). These facts also support the theory that many of the vibrios found in *Artemia* spp. cultures come from sea water and not from *Artemia* cysts.

Strains LMG 13240^T (= CAIM 525^T = VIB 213^T) and LMG 13213 (= CAIM 524 = VIB 186) were isolated from *Artemia* spp. during 1991. Strain LMG 13211 (= CAIM 523 = VIB 184) was isolated from sea water in 1990. All strains were isolated in a fish hatchery in Barcelona, Spain, as described elsewhere (Verdonck *et al.*, 1994). All strains have been deposited in the BCCM/LMG bacteria collection (Ghent, Belgium) and at the CAIM (Collection of Aquacultural Important Microorganisms; CIAD, A. C. Mazatlán, Mexico). These strains were previously analysed phenotypically (Austin *et al.*, 1995; Vandenberghe *et al.*, 2003) and with fluorescent AFLP (FAFLP) (Thompson *et al.*, 2001). The patterns obtained by Austin *et al.* (1995) with the Biolog GN system clustered only with those of another four strains of the 260 *Vibrio* spp. strains that were analysed; one of these strains belongs to the recently described species *Vibrio pacinii* (Gomez-Gil *et al.*, 2003), whereas Vandenberghe *et al.* (2003) clustered these strains only with

the type strain of *Vibrio gazogenes*. All of these strains were shown to be pathogenic to the Atlantic salmon (*Salmo salar*) and had unique ribotyping patterns (Austin *et al.*, 1995). Two of them (LMG 13213 and LMG 13240^T) also carried a 4.4 kbp plasmid (Austin *et al.*, 1995); analysis of these and many other *Vibrio* strains by FAFLP showed that these strains formed a unique cluster (A16; threshold delineation of 45%) that was not associated with any type strain included in the analysis (Thompson *et al.*, 2001).

Phenotypic characterization of the strains was performed as described previously (Gomez-Gil *et al.*, 2003). Antibiotic sensitivity was estimated by the disc diffusion test (Bauer *et al.*, 1966) on Iso-Sensitest agar (Oxoid) with 1.5% (w/v) NaCl. GLC analysis of methylated fatty acids was performed as described by Osterhout *et al.* (1991), but cells were grown on tryptic soy agar (TSA; Difco) with 1.5% (w/v) NaCl and incubated at 28 °C. Growth of the three strains at different NaCl concentrations was tested in a microtitre plate (five replicates) with peptone water (1.5% Bactopeptone; Difco); inoculated plates were incubated for 48 h at 30 °C and turbidity (OD₆₁₀) was measured.

Determination of the DNA G+C content of strain LMG 13240^T was performed as described by Mesbah *et al.* (1989) and modified by Logan *et al.* (2000). DNA–DNA hybridization was done following the methodology described by Willems *et al.* (2001) at 39 °C.

The 16S rRNA gene was amplified with primers V16S-9F (5'-AGAGTTTGATCATGGCTCAG-3') and V16S-1491R (5'-AGCGCTACCTTGTTACGACTT-3'). The PCR mix contained 18.46 µl water, 1.32 µl dNTP mix (2.5 mM each), 2.5 µl 10 × PCR buffer (with 25 mM MgCl₂), 0.26 µl each primer (0.25 µg µl⁻¹), 0.2 µl *Taq* (5 U µl⁻¹; Promega) and 2.0 µl DNA (final volume, 25 µl). Amplification parameters were 94 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and a final extension of 72 °C for 5 min, rendering a ~1.5 kbp product. Purified PCR products were ligated into a pGEM-T cloning vector (Promega). Transformation was performed by heat shock at 37 °C for 20 s by using competent cells of *Escherichia coli* DH5α (Gibco/Invitrogen). Cells were then incubated at 37 °C in SOC medium for 1 h, centrifuged, plated on Luria–Bertani medium (Invitrogen) that contained ampicillin (60 µg ml⁻¹) and incubated overnight at 37 °C. Plasmid extraction was performed by using a miniprep kit (Promega) according to the instructions of the manufacturer. Positive clones were identified by *Eco*RI digestion and gel electrophoresis. Bidirectional sequencing was carried out by using labelled T7/SP6 universal primers and a LiCor IR² DNA sequencer, as described by the manufacturer.

The 16S rRNA gene sequence of LMG 13240^T type 1 (GenBank accession no. AY254039) was also compared to the sequences deposited in GenBank (BLASTN; Altschul *et al.*, 1990) and the Ribosomal Database Project II (RDP; Sequence Match version 2.7; Cole *et al.*, 2003). Sequences

were aligned with CLUSTALX (version 1.8; Thompson *et al.*, 1997). Tree topology (neighbour-joining; Saitou & Nei, 1987) and stability of groupings (bootstrap analysis, 1000 replicates) of the closest phylogenetic species (with *Vibrio cholerae* El Tor strain as the outlier) and sequence similarities within the genus *Vibrio* (Jukes–Cantor model; $\Gamma = 0.4$; pairwise deletion; 1000 bootstrap replications; random number seed, 67 137) were calculated with the MEGA program (version 2.1; Kumar *et al.*, 2001).

Phenotypic characters, including fatty acid proportions (Bertone *et al.*, 1996), of the three strains analysed placed *Vibrio hispanicus* sp. nov. as a member of the genus *Vibrio* (see species description). Their phenotypic characteristics also permit clear differentiation from related species (Table 1), especially growth without NaCl, arginine dihydrolyase and lysine and ornithine decarboxylase.

Members of the genus *Vibrio* require Na⁺ for growth, with a few exceptions: *V. cholerae*, *Vibrio mimicus* and some strains of *Vibrio fluvialis*, *Vibrio furnissii* and *Vibrio metschnikovii* (Alsina & Blanch, 1994), although *V. fluvialis* and *V. furnissii* may need small amounts of Na⁺ to grow (Farmer & Hickman-Brenner, 1992). Optimal Na⁺ concentration for many marine bacteria is 70–300 mM (Reichelt & Baumann, 1974), which is well below that of sea water (450–480 mM), although many vibrios have been isolated from hypersaline environments (4185 mM), from which *Artemia* cysts are also harvested (Straub & Dixon, 1993). The strains analysed here were able to grow at 1.0 mM NaCl, but best growth was obtained at 1024 mM and no growth was detected at 2048 mM (see Supplementary Figure in IJSEM Online).

Table 1. Phenotypic differences between *V. hispanicus* sp. nov. and related arginine dihydrolyase-positive, lysine and ornithine decarboxylase-negative *Vibrio* species that grow in the absence of NaCl

Taxa are indicated as: 1, *V. hispanicus* sp. nov.; 2, *V. diazotrophicus*; 3, *V. fluvialis*; 4, *V. furnissii*; 5, *V. metschnikovii*. +, Positive; –, negative; (+), positive for 75–89% of strains; (–), negative for 25–11% of strains; v, variable. Data from Alsina & Blanch (1994) and Holt *et al.* (1994).

Test	1	2	3	4	5
Growth at:					
0% NaCl	+	–	v	v	v
4 °C	+	(+)	–	–	v
Indole	+	+	(+)	(–)	(–)
Oxidase	+	+	+	+	–
Gas from glucose	–	–	–	+	–
Resistance to O/129 (10 µg)	–	v	+	+	v
Gelatinase	–	–	+	(+)	+
Utilization of L-rhamnose	+	–	–	–	–

16S rRNA gene sequences of the strains analysed placed them in the genus *Vibrio*; their closest phylogenetic neighbours were *Vibrio proteolyticus* (97.6% BLASTN, 0.906 RDP), *Vibrio diazotrophicus* (97.9% BLASTN, 0.900 RDP), *Vibrio campbellii* (96.8% BLASTN, 0.857 RDP), *Vibrio alginolyticus* (96.8% BLASTN, 0.876 RDP), *Vibrio natriegens* (96.7% BLASTN, 0.867 RDP), *Vibrio parahaemolyticus* (96.6% BLASTN, 0.871 RDP), *Vibrio nigrispulchritudo* (96.6% BLASTN, 0.882 RDP), *Vibrio vulnificus* (97.1% BLASTN, 0.867 RDP) and *Vibrio harveyi* (96.4% BLASTN, 0.875 RDP). Phylogenetic analysis with the neighbour-joining method (Fig. 1) clustered *V. hispanicus* sp. nov. strains close to the *Vibrio* core group (Dorsch *et al.*, 1992). Strain LMG 13240^T produced two different 16S rRNA gene sequences, which differed by 15 bases (0.99%); five bases were within variable region (VR-) 1, five bases were within VR-7 and five bases were scattered in conserved regions.

DNA G + C content was 42.8 mol%, a value that lies within the range of the genus *Vibrio* (38–51%; Farmer, 1992). DNA–DNA similarities of strains LMG 13240^T and LMG 13211 with the species that was related most closely by 16S rRNA gene sequence similarity, *V. proteolyticus* LMG 3772^T, gave 22.5 and 22.7%, respectively. Hybridizations were performed with other closely related species: *V. harveyi* LMG 4044^T (24.7 and 26.4%, respectively), *V. nigrispulchritudo* LMG 3896^T (18.3 and 20.9%, respectively) and *V. natriegens* LMG 10935^T (23.9 and 24.2%, respectively). Hybridization was 102.5% between strains LMG 13240^T and LMG 13211 and 30.6% between *V. proteolyticus* LMG

3772^T and *V. harveyi* LMG 4044^T. The range of 16S rRNA gene sequence similarity within the *Vibrio* genus for *V. hispanicus* LMG 13240^T was 22–88%.

The data presented here, including phenotypic characteristics, 16S rRNA gene sequence analysis and DNA–DNA hybridization, and that of FAFLP fingerprinting that was published previously (Thompson *et al.*, 2001), clearly support the identity of strains LMG 13240^T, LMG 13211 and LMG 13213 as members of a novel species of the genus *Vibrio*, for which the name *Vibrio hispanicus* sp. nov. is proposed.

Description of *Vibrio hispanicus* sp. nov.

Vibrio hispanicus (his.pa'ni.cus. L. masc. adj. *hispanicus* from Spain).

Gram-negative, small ($2.2 \pm 0.659 \mu\text{m}$), motile, curved rods with polar flagellation. Bright yellow, small (1–3 mm), circular colonies are formed on TCBS agar. Non-luminescent, translucent and non-swarming colonies are formed on marine agar. Growth occurs in media that contain 0, 2.5, 6.0, 8.0 and 10% NaCl (w/v), but not in 12.0% NaCl. Growth occurs at 4, 30, 35 and 40 °C. Positive for oxidase, indole, citrate, methyl red, nitrite reduction and α -galactosidase. Fermentative, arginine dihydrolase-positive, lysine and ornithine decarboxylase- and L-tyrosine-negative. Tryptophan deaminase, H₂S, gas from glucose production, Voges–Proskauer reaction, gelatinase and urease are negative. Susceptible to vibriostatic agent O/129 at 10 and 150 μg and to polymyxin B at 300 U, but resistant to streptomycin (25 μg) and gentamicin (10 μg). Positive for α -D-glucose, β -hydroxybutyric acid, methyl β -D-glucoside, D-glucuronic acid (LMG 13213 is weakly positive), cellobiose, DL-lactic acid, dextrin, D-fructose, D-galactose, D-gluconic acid, D-mannitol, D-mannose, D-raffinose (LMG 13240^T and LMG 13213 are weakly positive), D-saccharic acid (LMG 13213 is weakly positive), D-trehalose, gentiobiose, inosine, L-asparagine, L-aspartic acid (LMG 13240^T is weakly positive), L-glutamic acid, L-rhamnose, L-serine, maltose, methyl pyruvate, N-acetyl-D-glucosamine, psicose, sucrose, thymidine and uridine utilization as sole carbon sources. Negative for 2,3-butanediol, 2-aminoethanol, acetic acid, adonitol, alaninamide, α -cyclodextrin, α -D-lactose lactulose, α -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, bromosuccinic acid, *cis*-aconitic acid, citric acid, DL- α -glycerol phosphate, DL-carnitine, D-alanine, D-arabitol, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-melibiose, D-serine, D-sorbitol, formic acid, γ -aminobutyric acid, γ -hydroxybutyric acid, glucose 1-phosphate, glucose 6-phosphate, glucuronamide, glycogen, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy L-proline, i-erythritol, itaconic acid, L-alanine, L-alanylglycine, L-fucose, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyrogutamic acid, L-threonine, malonic acid, *myo*-inositol, monomethyl succinate, N-acetyl-D-galactosamine, phenylethylamine, *p*-hydroxyphenylacetic acid, propionic acid,

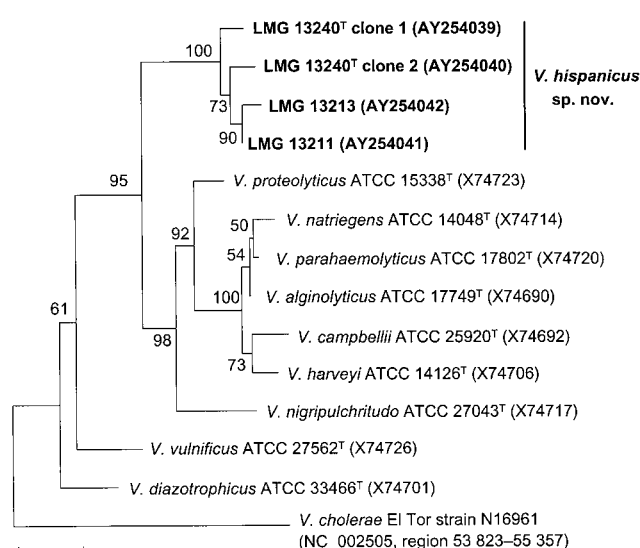


Fig. 1. Phylogenetic dendrogram of *Vibrio hispanicus* sp. nov. strains with the most closely related *Vibrio* species, based on almost-complete 16S rRNA gene sequences (1519 bp) and constructed by the neighbour-joining method (pairwise deletion, Kimura two-parameter, $\Gamma = 0.4$). Numbers at nodes indicate level of bootstrap support (1000 replicates). Bar, 1% divergence.

putrescine, quinic acid, sebacic acid, succinamic acid, turanose, Tween 40, Tween 80, urocanic acid and xylitol as sole carbon sources. Acid and alkaline phosphatases, β -glucosidase, esterase, esterase lipase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase are positive; α -chymotrypsin, α -fucosidase, α -glucosidase, α -mannosidase, β -galactosidase, β -glucuronidase, cystine arylamidase, lipase, *N*-acetyl- β -glucosaminidase, trypsin and valine arylamidase are negative. Most abundant fatty acids (% of total fatty acids) are, in descending order (mean of three strains, minimum and maximum): summed feature 3 ($C_{16:1\omega7c}$ and/or iso- $C_{15:0}$ 2-OH; 37.2, 35.8–39.1), $C_{16:0}$ (25.7, 22.8–27.9), $C_{18:1\omega7c}$ (16.7, 15.5–17.8), $C_{14:0}$ (5.9, 5.3–6.5), $C_{12:0}$ (4.4, 4.2–4.8), summed feature 2 ($C_{14:0}$ 3-OH and/or iso- $C_{16:1}$ I; 3.2, 3.1–3.4), $C_{12:0}$ 3-OH (2.6, 2.5–2.8) and $C_{18:0}$ (1.1, 0.9–1.2). Differences between strains analysed are: LMG 13240^T does not utilize α -lactose, LMG 13211 does not utilize glycerol or amygdalin and LMG 13213 does not utilize succinic acid. LMG 13213 is sensitive to ampicillin (30 μ g; the others are intermediately resistant) and is intermediately resistant to kanamycin (30 μ g; the others are resistant) and oxytetracycline (30 μ g; the others are resistant); LMG 13211 is resistant to amikacin (30 μ g; the others are intermediately resistant). DNA G+C content is 42.8 mol%.

The type strain is LMG 13240^T (=CAIM 525^T=VIB 213^T) and reference strains are LMG 13211 (=CAIM 523) and LMG 13213 (=CAIM 524). All were isolated from *Artemia* sp. and its culture water in Barcelona, Spain.

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