Aliivibrio finisterrensis sp. nov., isolated from Manila clam, *Ruditapes philippinarum* and emended description of the genus *Aliivibrio*

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Four strains isolated from cultured Manila clam, *Ruditapes philippinarum*, in the north-western coast of Spain were characterized phenotypically and genotypically. Phylogenetic analyses based on the 16S rRNA gene sequences indicated that these bacteria were closely related to *Aliivibrio wodanis*, *Aliivibrio salmonicida*, *Aliivibrio fischeri* and *Aliivibrio logei* with sequence similarities between 98.1 and 96.0%. Phylogenetic analysis based on the RNA polymerase alpha chain (*rpoA*), RecA protein (*recA*), the α -subunit of bacterial ATP synthase (*atpA*) and the uridine monophosphate (UMP) kinase (*pyrH*) genes and fluorescent amplified fragment length polymorphism experiments clearly showed that these novel isolates form a tight genomic group different from any currently known *Aliivibrio* species. On the basis of phylogenetic analysis and phenotypic data, the four strains represent a novel taxon, for which the name *Aliivibrio finisterrensis* sp. nov. is proposed. Several phenotypic features were revealed that discriminate *A. finisterrensis* from other *Aliivibrio* species. The type strain is CMJ 11.1^T (=CECT 7228^T=LMG 23869^T).

The controversy on the taxonomic position and phylogenetic relations of the *Vibrio fischeri* group, located between the genus *Vibrio* and *Photobacterium* (Sawabe *et al.*, 2007), originated in the 1970s and has remained for decades (Reichelt & Baumann, 1973; Baumann & Baumann, 1977; Baumann *et al.* 1980; Thysen & Ollevier, 2005). A recent study by Urbanczyk *et al.* (2007) demonstrated the phylogenetical and phenotypical distinction of the group from other genera in the family *Vibrionaceae* and proposed the novel genus *Aliivibrio*. The genus *Aliivibrio* contains four species: *Aliivibrio fischeri* and three psychrotolerant species, *Aliivibrio salmonicida*, *Aliivibrio wodanis* and

Aliivibrio logei. *A. fischeri*, originally isolated by Beijerinck *et al.* (1889) (Urbanczyk *et al.* 2007), is known for the light organ symbiosis with the Hawaiian bobtail squid *Euprymna scolopes* and has been studied as a model for vibrio–host interactions. Bacterial bioluminescence acts as an anti-predatory mechanism and serves researchers as a way to monitor bacterial colonization (Stabb, 2006). *A. salmonicida* described by Egidius *et al.* (1986), is a psychrophilic species known as the causative agent of cold water vibriosis (Colquhoun & Sørum, 2001) or the Hitra disease affecting Atlantic salmon (*Salmo salar*) (Egidius *et al.*, 1981). *A. wodanis* (Lunder *et al.*, 2000) also affects Atlantic salmon and has been associated with the winter ulcer disease (Lunder *et al.*, 1995). *A. logei* shares characteristics with the other members of the genus. The bacterium is bioluminescent, has the ability to associate with salmonids reared at low temperatures and causes the Hitra disease (Benediktsdottir *et al.*, 1998), but also resides symbiotically in light organs of several species of Mediterranean sepiolid squids. At lower temperatures (15 °C) it is the dominant symbiont, whereas at higher temperatures (26 °C) it is out-competed by *A. fischeri* (Fidopiastis *et al.*, 1998; Nishiguchi, 2000).

Aliivibrio related species and their hosts have been considered as models of either a co-evolution symbiosis or a pathogenic disease. To understand the driving force of the genetic diversity and speciation of *A. fischeri* related

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; NJ, neighbour-joining; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA*, *rpoA*, *atpA* and *pyrH* gene sequences of *A. finisterrensis* strains CMJ 11.1^T, C 13.19, C 17.13 and VB 14.7 (CECT 7356) are EU541604, EU541586, EU541567, EU541561, EU889126, EU541614, EU541603, EU541583, EU541562, EU889127, EU541616, EU541598, EU541584, EU541563, EU889129, EU541615, EU931115–EU931117 and EU889128, respectively. The GenBank/EMBL/DDBJ accession number for the *atpA* gene sequence of *Aliivibrio salmonicida* NCIMB 2262^T is EU541564.

Transmission electron micrograph of *A. finisterrensis* sp. nov., protein profile of the type strain of *Vibrio gallaecicus*, CMJ 11.1^T obtained by the technique MALDI-TOF MS and three additional phylogenetic trees are available with the online version of this paper.

species is important to isolate novel species within this genus. In this study, we examined four strains from cultured Manila clams, *Ruditapes philippinarum*. Phylogenetic analysis and phenotypic characterization demonstrated that these strains represent an unknown species of the genus *Aliivibrio*.

During a sampling programme performed in the years 2004 and 2005, a collection of marine bacterial strains were isolated from different geographical sites on the north-western coast of Spain. In a previous study, a representative number of isolates of this collection were analysed by amplified fragment length polymorphism and one of the clusters (cluster 86) with four strains [CMJ 11.1^T (=CECT 7228^T=LMG 23869^T), VB 14.7 (=CECT 7356), C 13.19, C 17.13] could not be assigned to any of the currently known *Vibrionaceae* species (Beaz-Hidalgo *et al.*, 2008). All strains were cultured on plates of marine agar (MA; Pronadisa) and were grown at 25 ± 1 °C for 24 h. The strains *A. salmonicida* NCIMB 2262^T, *A. fischeri* LMG 4414^T, *A. wodanis* ATCC BAA-104^T and *A. logei* LMG 19806^T were also used in this study. Strains of *A. wodanis* (ATCC BAA-104^T) and *A. salmonicida* (NCIMB 2262^T) were grown at 15 °C for 24 h. Stock cultures were maintained frozen at -80 °C in marine broth (Pronadisa) supplemented with 15 % glycerol (v/v). Biochemical characterization of the four clam isolates was performed by standard techniques, following the methodologies described by Lemos *et al.* (1985), Mac Faddin (1993), West *et al.* (1986) and Romalde & Toranzo (1991). Further phenotypic characterization was performed using API 20E and API ZYM (bioMérieux) following the instructions of the manufacturer, with the exception of using saline solution (0.85 %) for the preparation of the inocula. Acid production of 50 carbon sources was tested by using the API 50CH system with slight modifications. Briefly, bacterial suspensions were prepared in saline solution (0.85 % NaCl), adjusted to an optical density of 1.0 at 580 nm and mixed (1:90, v/v) with the ZOF medium (Lemos *et al.*, 1985) for inoculation procedures (Prado, 2006).

Flagellar arrangement and cellular size were determined by transmission electron microscopy (TEM). For TEM, cells were stained with fosfotungstic acid (2 %) and processed samples were visualized by an electron microscope Philips CM-12.

Genomic DNA extraction, amplification and sequencing reactions of the 16S rRNA gene were performed as previously described (Osorio *et al.*, 1999). Sequencing of the genes *rpoA*, *recA*, *atpA* and *pyrH* was carried out by the methods described by Thompson *et al.* (2004, 2005, 2007). Sequencing reactions were performed using the kit GenomeLab DTCS-Quick Start kit (Beckman Coulter). The 16S rRNA gene and four housekeeping genes were sequenced for all four strains. Sequencing was also performed for the strain *A. salmonicida* NCMB 2262^T for the *atpA* gene due to its absence in the database. Sequence corrections and analysis were performed with DNASTAR

Seqman program (Lasergene). Sequences were retrieved and levels of gene sequence similarities were determined by using the EzTaxon (<http://www.eztaxon.org/>; Chun *et al.*, 2007), leBIBI (<http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi>; Devulder *et al.*, 2003) and NCIMB/BLAST (<http://blast.ncbi.nlm.nih.gov/>) release servers. Phylogenetic relationships between representatives of *Vibrio* and *Aliivibrio* species were determined by using MEGA version 4.0 software program (Tamura *et al.*, 2007). Distance matrices were determined (Kimura, 1980) and these matrices were used to elaborate dendrograms by using the neighbour-joining (NJ) method (Saitou & Nei, 1987). A bootstrap analysis to investigate the stability of the trees was performed on 1000 replicates.

DNA-DNA hybridization experiments, including reciprocal analysis, were done in duplicate by the hydroxyapatite/microtitre plate method (Ziemke *et al.*, 1998) with a hybridization temperature (T_m) of 60 °C.

The protein analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was performed in the mass spectrometry unit of the University of Santiago de Compostela. Protein extraction was performed with ethanol, formic acid and acetonitrile. Processed samples were placed in a 96-well plate, allowed to dry and covered with a matrix solution (α -cyano-4-hydroxycinnamic acid). Mass spectra were obtained using a MALDI-TOF Autoflex mass spectrometer (Bruker Daltronics). The measured mass range of spectra was 2000–20 000 Da. Peak comparison was done with the database of Bruker Daltronics. The species-limit value considered was 2300. Identification to genus level was in the range 1700–1999. As a positive control *Escherichia coli* CECT 433 was included in the analysis and protein profiles were compared with its own profiles.

Cells of the novel *Aliivibrio* species were large rods of size 1.36–2.15 µm in length and 0.80–1.05 µm in width and occurred singly, in pairs or as aggregates of many cells. The presence of a polar flagellum was observed by TEM (Supplementary Fig. S1, available in IJSEM Online).

All strains isolated were motile, facultative anaerobic, Gram-negative, oxidase-positive rods and susceptible to the vibriostatic agent O/129 (150 µg per disc). They were able to grow only in media supplemented with NaCl (from 0.5 to 6 %) between 4 and 30 °C, and reduced nitrates to nitrites. The four clam strains were phenotypically homogeneous, with six variable traits, such as production of acid from arbutin, D-mannitol, sucrose and melibiose, with strain C 17.13 being more reactive than the rest of the isolates. Several useful differentiating features were disclosed that discriminate this group of isolates from other related *Aliivibrio* and *Vibrio* species (Table 1). All strains failed to grow on media supplemented with 0.5 % NaCl and were positive for Moeller's arginine dihydrolase and the urea test unlike the other four *Aliivibrio* species.

Table 1. Differential phenotypic characteristics of strain *A. finisterrensis* and the type strains of related *Aliivibrio* and *Vibrio* species

Taxa (species/strains) are indicated as: 1, *A. finisterrensis* CMJ 11.1^T (=CECT 7228^T=LMG 23869^T) (four strains); 2, *A. fischeri* NCMB 1281^T; 3, *A. wodanis* ATCC BAA-104^T; 4, *A. logei* LMG 19806^T; 5, *A. salmonicida* NCMB 2262^T; 6, *Vibrio aestuarianus* ATCC 35048^T; 7, *Vibrio mediterranei* CECT 621^T; 8, *Vibrio metschnikovii* ATCC 7708^T; 9, *Vibrio orientalis* CECT 629^T; 10, *Vibrio proteolyticus* ATCC 15338^T. +, Positive; –, negative; v –, variable but type strain is negative (percentage of strains testing positive are in parentheses). All strains were positive for catalase, oxidase, motility, growth at 15 °C, growth in 3 % NaCl, susceptibility to the vibriostatic agent and ampicillin, degradation of DNA, fermentation of D-glucose and acid production from alkaline phosphatase. All strains were negative for Gram stain, production of ornithine decarboxylase, degradation of alginate, acid production from inositol, L-arabinose, melibiose, raffinose, L-rhamnose, D-sorbitol and D-xylose, production of cysteine arylamidase, α -chymotrypsinase, α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase and α -fucosidase.

Characteristic	1	2	3	4	5	6	7	8	9	10
Production of										
Arginine dihydrolase	+	–	–	–	–	+	+	+	–	+
Lysine decarboxylase	+	+	–	+	–	+	+	+	+	+
Indole	–	+	+	–	–	+	+	–	+	+
Urea	+	+	–	–	–	–	–	–	–	–
Voges–Proskauer	–	+	–	–	–	–	–	+	–	+
Methyl red test	+	–	+	+	–	+	+	–	+	–
Degradation of:										
Starch	–	–	+	–	–	+	+	+	+	+
Gelatin	–	–	+	–	–	+	–	+	+	–
Tween 80	+	+	+	+	–	+	+	+	+	+
Production of acid from:										
Cellobiose	+	–	–	+	–	+	+	–	+	+
Glycerol	+	–	+	+	+	+	+	+	+	+
D-Mannitol	v– (25)	+	–	+	+	+	+	+	+	+
D-Mannose	+	–	+	+	+	+	+	+	+	+
Salicin	+	+	–	+	–	–	+	–	–	–
Sucrose	v– (25)	+	+	–	–	+	+	+	+	–
Trehalose	–	–	+	+	+	+	+	+	+	+
Growth in NaCl (% w/v):										
0.5	–	+	+	+	+	–	+	+	+	+
8	–	+	–	–	–	+	+	–	+	+
Growth at (°C):										
4	+	–	+	+	+	+	–	–	+	–
25	+	+	+	–	–	+	+	+	+	+
30	+	+	–	–	–	+	+	+	+	+
35	–	+	–	–	–	–	–	+	+	+
API ZYM tests:										
Esterase (C4)	v– (25)	–	+	+	+	–	–	–	–	+
Leucine arylamidase	+	+	+	+	–	+	+	+	+	+
Acid phosphatase	–	+	v	+	+	+	+	–	+	+
Naphthol phosphohydrolase	–	+	+	+	+	+	+	–	+	+

The results of the phylogenetic analysis based on the 16S rRNA gene showed clearly that the strains belong to subgroup 3 of the class *Gammaproteobacteria*. The phylogenetic tree based on the 16S rRNA gene sequences confirmed the clustering of the clam strains CMJ 11.1^T, VB 14.7, C 13.19 and C 17.13, with a bootstrap value of 100 %, and their distinction from their closest phylogenetic neighbours (Supplementary Fig. S2, available in IJSEM Online). These strains showed high levels of 16S rRNA gene sequence similarity to each other above 99.9 %. Gene sequence similarities of the strain CMJ 11.1^T with close phylogenetic neighbours were 98.1 % with *A. wodanis* (ATCC BAA-104^T), 97.8 % with *A. logei* (LMG 19806^T),

97.1 % with *A. fischeri* (LMG 4414^T) and 96.0 % with *A. salmonicida* (NCMB 2262^T).

The clustering of the novel species isolates was also observed in phylogenetic trees based on the housekeeping genes (Supplementary Fig. S3, available in IJSEM Online). Similarities for *rpoA* sequences ranged between 98.1 (CMJ 11.1^T and *A. wodanis* ATCC BAA-104^T) and 97.2 % (CMJ 11.1^T and *A. fischeri* LMG 4414^T). For *recA* sequences, similarities ranged from 89.0 (CMJ 11.1^T and *A. fischeri* LMG 4414^T) to 81.0 % (CMJ 11.1^T and *A. salmonicida* NCMB 2262^T), whereas for *atpA* sequences similarities ranged from 95.1 (CMJ 11.1^T and *A. wodanis* ATCC BAA-

104^T) to 92.0 % (CMJ 11.1^T and *A. salmonicida* NCMB 2262^T). Finally, similarities in *pyrH* sequences ranged between 90.0 (CMJ 11.1^T with *A. wodanis* ATCC BAA-104^T and *A. salmonicida* NCMB 2262^T) and 87 % (CMJ 11.1^T and *A. fischeri* LMG 4414^T). A concatenated tree based on the 16S rRNA gene and four housekeeping genes revealed a robust clade of the strains analysed in this study with 100 % bootstrap value, not including any species with validly published names (Fig. 1 and Supplementary Fig S4, available in IJSEM Online).

DNA–DNA hybridization values of strain CMJ 11.1^T against the four species in the genus were 32.8 % with *A. fischeri* LMG 4414^T, 32.4 % with *A. salmonicida* NCMB 2262^T, 46.5 % with *A. logei* LMG 19806^T and 66.5 % with *A. wodanis* ATCC BAA-104^T, being below the 70 % threshold established to delineate a novel species (Wayne *et al.*, 1987). Reciprocal analysis rendered values of hybridization of 65.5, 41.6, 34.6 and 27.5 % between *A. wodanis* ATCC BAA-104^T, *A. logei* LMG 19806^T, *A. salmonicida* NCMB 2262^T and *A. fischeri* LMG 4414^T, with CMJ11.1^T, respectively. Hybridization values between the type strain CMJ 11.1^T, and the rest of the strains C 13.19, C 17.13 and VB 14.7 were 80.1, 90.0 and 86.5 %, respectively.

A unique protein profile was obtained for the strain CMJ 11.1^T (Supplementary Fig. S5, available in IJSEM Online), differentiating them from closely related species present in the database. The closest species defined by MALDI-TOF MS was *A. fischeri* (score=1.671). A correct identification of *E. coli* CECT 433 was obtained, with profiles always above 2.300 in score.

Genotypic and phenotypic data described in this study clearly demonstrate that the four isolates should be accommodated in a novel species in the genus *Aliivibrio* namely *Aliivibrio finisterrensis* with the strain CMJ 11.1^T as the type strain.

Description of *Aliivibrio finisterrensis* sp. nov.

Aliivibrio finisterrensis [fi.nis.ter.ren'sis. N.L. masc. adj. *finisterrensis* pertaining to Finisterra, literally the end of the world (at least for the Romans who named this place). Galicia was the western end of the ancient Roman world].

Cells are Gram-negative rods, motile and facultative anaerobic. Colonies are round with smooth margins, translucent and non-swarming on MA plates. Strains grow on TCBS agar producing colonies of size 1.0–1.5 mm after 24 h of incubation. One strain (C 17.13) produces cream colonies on MA and yellow colonies on TCBS, the other strains produce green colonies. Glucose metabolism is fermentative without gas production. Strains reduce nitrates to nitrites. Oxidase, catalase and ONPG tests are positive. Sensitive to the vibriostatic agent O/129 (150 µg per disc) and resistant to ampicillin (10 µg per disc), and are able to grow at 4 °C but not at 35 °C nor at 0.5 or 8 % NaCl (w/v). Optimal NaCl concentration for growth is 1–3 % (w/v). Not luminescent. All strains are positive for arginine and lysine decarboxylase, urea and methyl red tests. Tests for ornithine decarboxylase, indole, Voges–Proskauer and use of citrate are negative. Strains do not hydrolyse starch, gelatin or alginate. All strains are positive for DNase, Tween 80 and aesculin activities. Acid is produced from the following compounds: glycerol, D-glucose, D-ribose, D-galactose, D-fructose, D-mannose, N-acetylglucosamine, salicin, cellobiose, maltose, lactose (bovine origin), gentiobiose and potassium 2-ketogluconate. Half of the strains produce acid from arbutin and melibiose. Strain CMJ 11.1^T and C 13.19 are positive for the production of acid from arbutin, type strain only also for melibiose, and strain C 17.13 for D-mannitol and sucrose. None of the strains produce acid from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulci-

Fig. 1. Phylogenetic tree based on concatenated sequences of four housekeeping genes *recA*, *rpoA*, *pyrH* and *atpA* and the 16S rRNA gene obtained by the NJ method. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap percentages from 1000 replicates appear next to the corresponding branch. A similar topology was found by the maximum-likelihood method (see Supplementary Fig. S4).

tol, inositol, starch, glycogen, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, amygdalin, trehalose, inulin, melezitose, D-raffinose, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and potassium 5-ketogluconate. API ZYM reaction tests were positive in all strains for alkaline phosphatase, esterase lipase, leucine arylamidase and N-acetyl- β -glucosaminidase. Strain C 17.13 also showed positive results for esterase (C4) and valine arylamidase.

The type strain, CMJ 11.1^T (=CECT 7228^T=LMG 23869^T), was isolated from clam *Ruditapes philippinarum* in the north-western coast of Spain.

Emended description of the genus *Aliivibrio*

Gram-negative, motile, rod-shaped cells with one or more sheathed flagella. Conforms to the description of the family *Vibrionaceae*. Some strains are luminous. Oxidase-positive, fermentative and can utilize glucose as a sole carbon source; sensitive to the vibriostatic agent O/129 at 10 μ g. Grow on media with 1 %, but not with 10 % NaCl. Species are ornithine decarboxylase-negative, do not hydrolyse gelatin and do not form acetoin (Voges-Proskauer test). *A. fischeri* and *A. finisterrensis* are urease-positive and ferment gentiobiose; all other species of the genus are negative for these two characteristics. All species utilize acetate as a sole carbon source except *A. fischeri*. Species other than *A. salmonicida* and *A. finisterrensis* have yellow-orange cell-associated pigment. The DNA G + C content is between 38 and 42 mol%. Found in the marine environment, often associated with animals; some species are mutualistic symbionts or pathogens of marine animals. Member of the class *Gammaproteobacteria*. The type species is *Aliivibrio fischeri*.

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