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Vibrio atlanticus sp. nov. and *Vibrio artabrorum* sp. nov., isolated from the clams *Ruditapes philippinarum* and *Ruditapes decussatus*

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Facultatively anaerobic marine bacteria isolated from cultured clams, *Ruditapes decussatus* and *Ruditapes philippinarum*, were previously investigated using amplified fragment length polymorphism (AFLP) and 16S rRNA gene sequence analyses. The isolates formed two AFLP clusters and belonged to the genus *Vibrio*, more precisely to the Splendidus clade. In this study, phylogenetic analyses based on sequences of the housekeeping genes *rpoA*, *rpoD*, *pyrH*, *atpA* and *recA* supported their inclusion in that clade forming two well differentiated groups with respect to the rest of the species within the clade, and confirmed that they formed two groups, separated from the rest of the species of the clade. DNA–DNA hybridization demonstrated that the isolates constitute two novel species of the genus *Vibrio*, which can be phenotypically differentiated from their closest relatives. The names *Vibrio atlanticus* sp. nov. and *Vibrio artabrorum* sp. nov. are proposed, with Vb 11.11^T (=CECT 7223^T =LMG 24300^T) and Vb 11.8^T (=CECT 7226^T =LMG 23865^T) as the type strains, respectively.

Vibrios were one of the first groups of bacteria to be recognized in nature (Pacini, 1854). Their taxonomy has been studied in depth, and has been subjected to consecutive rearrangements as a result of the development and use of molecular techniques such as DNA–DNA hybridization, multilocus sequence analysis (MLSA) and amplified fragment length polymorphism (AFLP), among others (Colwell, 2006; Thompson & Swings, 2006; Beaz-Hidalgo *et al.*, 2010). Moreover, the great abundance of vibrios in aquatic environments, the high diversity detected among members of the family *Vibrionaceae* and the increasing number of environmental studies have significantly contributed to the description of novel species of the genus *Vibrio* (Austin *et al.*, 1997; Beaz-Hidalgo *et al.*, 2010). At the time of writing, more than 70 species are recognized, grouped in 14 clades (Sawabe *et al.*, 2007). Among these, the Splendidus clade contains the highest number of species (>10), which have been found to be the dominant *Vibrio* species in coastal marine sediments, seawater and bivalves in temperate climates (Lambert *et al.*, 1998; Sobecky *et al.*, 1998). Some of these species have been associated with mortality of a wide range of marine animals such as molluscs, crustaceans and fish (Kueh & Chan, 1985; Pujalte *et al.*, 1993; Nicolas *et al.*, 1996; Leano *et al.*, 1998; Sugumar *et al.*, 1998; Lacoste *et al.*, 2001; Le Roux *et al.*, 2002; Waechter *et al.*, 2002; Farto *et al.*, 2003; Jensen *et al.*, 2003; Gay *et al.*, 2004; Gómez-León *et al.*, 2005).

In a previous study, a collection of isolates tentatively allocated to the genus *Vibrio* based on their phenotypic features were analysed by AFLP. Two groups, cluster 5 and cluster 70, consisted of isolates that could not be assigned to any of the currently known species of the genus *Vibrio* (Beaz-Hidalgo *et al.*, 2008). In this study, we report on the further taxonomic characterization of these two groups of isolates obtained from clams, *Ruditapes philippinarum* and *Ruditapes decussatus*, cultured in Galicia.

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Abbreviations: AFLP, amplified fragment length polymorphism; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; VP, Voges–Proskauer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rec*A, *rpo*A, *rpo*D, *atp*A and *pyr*H gene sequences of strain Vb 11.11^T are EF599163, EU541589, EU541569, FN582265, FN582252 and FN582266, respectively. Those for the 16S rRNA, *rec*A, *rpo*A, *rpo*D, *atp*A and *pyr*H gene sequences of strain Vb 11.8^T are EF599164, EU541588, EU541568, FN668926, FN668898 and FN668929, respectively. Accession numbers for sequences of the other isolates studied are given in Supplementary Tables S1 and S2.

Seven supplementary tables and seven supplementary figures are available with the online version of this paper.

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Cluster 5 isolates [Vb 11.11^{T} (=CECT 7223^{T} =LMG 24300^{T}), C 14.7 (=LMG 25697), C 2.4, C 15.19 and Cmj 13.4] and cluster 70 isolates [Vb 11.8^{T} (=CECT 7226^{T} =LMG 23865^{T}), Cmf 5.9, Cmf 6.5 (=LMG 25698), Cmf 11.16, Rd 16.2, Rd 14.14, Rd 14.3 and C 15.18] were obtained from cultured clams on the north-west coast of Spain (Galicia) during a two-year survey from March 2004 to December 2005 as previously described (Beaz-Hidalgo *et al.*, 2008). The type strains *Vibrio gigantis* LMG 22741^{T}, *V. tasmaniensis* LMG 20012^{T}, *V. kanaloae* LMG 20539^{T}, *V. cyclitrophicus* LMG 21359^{T}, *V. crassostreae* LMG 22240^{T}, *V. pomeroyi* LMG 20537^{T}, *V. splendidus* LMG 19031^{T}, *V. lentus* CECT 5110^{T}, *V. gallaecicus* LMG 24045^{T} (=CECT 7244^{T}) and *V. chagasii* LMG 21353^{T} were obtained from the BCCM/LMG collection and included in the study for taxonomic comparison. All strains were cultured on marine agar (MA, Pronadisa) at 25 °C for 24 h. Cultures were maintained frozen at –80 °C in marine broth (MB, Pronadisa) supplemented with 15 % (v/v) glycerol.

Phenotypic characteristics were determined using standard methods and by commercial miniaturized kits (API 50CH, API 20E and API ZYM; bioMérieux). API 50CH was used with slight modifications as reported previously (Beaz-Hidalgo *et al.*, 2009). Briefly, bacterial suspensions were prepared in saline solution (SS; 0.85 % NaCl), adjusted to an OD_{580} of 1.0 and mixed with ZOF medium (Lemos *et al.*, 1985) (1 : 10, v/v) prior to the inoculation of the galleries. Readings were taken at 24, 48, 96 and 120 h and after 6 days. Readings after 6 days of incubation were considered as the final result. For API 20E and API ZYM, standard methodologies were used except that the SS was used to prepare the bacterial suspensions. In all cases, incubation was done at 25 °C. Routine phenotypic tests were performed following the methodologies described by Lemos *et al.* (1985), West *et al.* (1986), Romalde & Toranzo (1991) and MacFaddin (1993). All media were supplemented with 1 % NaCl when required.

Genomic DNA for sequencing was extracted as described previously (Prado *et al.*, 2005). Amplification and sequencing of the 16S rRNA gene were performed by using a GenomeLab DTCS-Quick Start kit (Beckman Coulter). Amplification and sequencing of the housekeeping genes *rpoA*, *recA*, *atpA*, *pyrH* and *rpoD* were performed according to Thompson *et al.* (2004, 2005, 2007). Accession numbers for sequences of the isolates studied are given in Supplementary Tables S1 and S2 (available in IJSEM Online). For reference strains, sequences were retrieved from GenBank/EMBL/DDBJ. Sequence data analysis was performed with the DNASTAR Lasergene SEQMAN program. Sequences of the isolates were subjected to a BLAST search against the latest release of GenBank. Phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood algorithms (Tamura *et al.*, 2007; Posada, 2008; FigTree v1.1.2, http://tree.bio.ed.ac.uk/). Distance matrices were calculated using Kimura's two-parameter correction. Stability of the groupings was estimated by bootstrap analysis (1000 replicates) using the program MEGA version 4.0 (Tamura *et al.*, 2007). If needed, putative recombination events were detected using RDP3 Beta 41 (Martin *et al.*, 2005).

All strains of both clusters were facultatively anaerobic, Gram-negative, and oxidase and catalase-positive. All isolates from cluster 5 were motile, but in cluster 70 only three out of eight isolates showed this characteristic. They required salt for growth, grew on thiosulfate-citrate-bile-sucrose agar (TCBS, Oxoid), reduced nitrates to nitrites, and were able to grow at 4 °C; features typical for vibrios. Phylogenetic analysis based on 16S rRNA gene sequences confirmed that the isolates belonged to the genus *Vibrio*. They were found to be related to species of the Splendidus clade (Supplementary Figs S1 and S2). Isolate Vb 11.11^{T} (cluster 5) showed highest sequence similarities with the species *V. tasmaniensis* (99.3 %), *V. lentus* (99.1 %) and *V. splendidus* (99.0 %) (Supplementary Table S3), whereas *V. gigantis* (99.9 %), *V. pomeroyi* (99.4 %), *V. crassostreae* (99.5 %), *V. tasmaniensis* (99.2 %) and *V. splendidus* (99.0 %) were the closest relatives of isolate Vb 11.8^{T} (cluster 70) (Supplementary Table S4).

Sequence analysis of housekeeping genes has proven to be a useful tool to infer phylogenetic relationships among micro-organisms. For the family *Vibrionaceae*, different loci, including *gyrB*, *recA*, *rpoA*, *pyrH*, *atpA* and *dnaJ*, have been studied in the search for a phylogenetic marker capable of delineating species of the genus *Vibrio* (Thompson *et al.*, 2004, 2005, 2007; Sawabe *et al.*, 2007; Nhung *et al.*, 2007; Pascual *et al.*, 2010). In the present study, sequences of the genes *recA* (613–713 bp), *rpoA* (931 bp), *pyrH* (443 bp), *atpA* (1322 bp) and *rpoD* (780 bp) were obtained for the clam isolates and compared with those of related species (Supplementary Tables S3 and S4). Based on the housekeeping gene sequences analysed, with the exception of the *rpoD* and *atpA* genes, *V. tasmaniensis* appeared to be the closest relative of the cluster 5 isolates (Supplementary Table S3), with similarity values higher than the interspecies limit previously described (Thompson *et al.*, 2005). However, the inaccuracy of these limits has been pointed out recently by Pascual *et al.* (2010) for species belonging to the central clade of the genus *Vibrio*. In addition, within cluster 5 isolates, recombination events have been detected in the *pyrH* and *recA* genes for strains C 15.19 and C 14.17, respectively. These recombination events affect the trees' topologies (Supplementary Fig. S3). In the case of the cluster 70 isolates, each housekeeping gene pointed a different species of the genus *Vibrio* as the closest relative (i.e. *Vibrio kanaloae* with *recA* or *Vibrio pomeroyi* with *rpoA*) (Supplementary Table S4 and Supplementary Fig. S4). Phylogenetic trees based on concatenated sequences of the 16S rRNA, *rpoA*, *recA*, *pyrH*, *atpA* and *rpoD* genes confirmed the allocation of the isolates in the Splendidus clade, also enabling their differentiation from the recognized species of the clade (Figs 1 and 2, Supplementary Figs S5 and S6).

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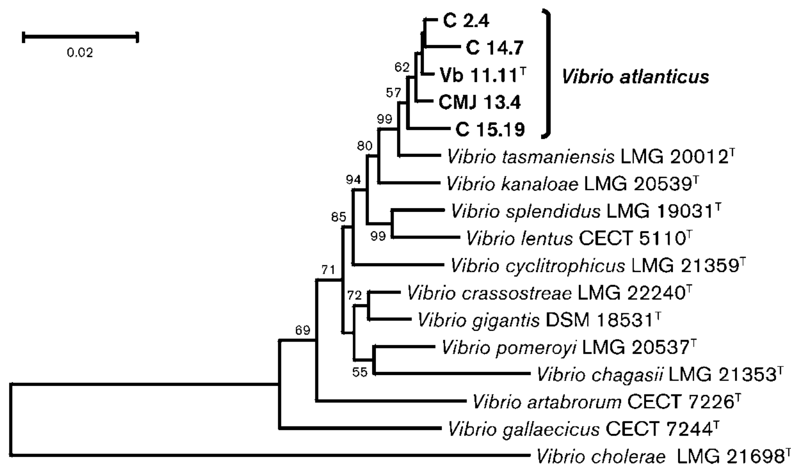

Fig. 1. Neighbour-joining tree showing the phylogenetic position of the cluster 5 strains, based on concatenated sequences of the four housekeeping genes *recA*, *rpoA*, *rpoD* and *atpA*, and the 16S rRNA gene. *Vibrio cholerae* was used as the outgroup. The stability of the grouping was estimated by bootstrap percentages from 1000 replicates. Similar results were obtained with the maximum-likelihood algorithm. Bar, 0.02 substitutions per nucleotide position. Numbers at nodes show percentage bootstrap values.

Genomic DNA for DNA–DNA hybridization experiments and DNA G+C content determinations was extracted as previously described (Cleenwerck *et al.*, 2002). DNA–DNA hybridizations between strains Vb 11.11^{T}, Vb 11.8^{T} and the type strains of the known species of the Splendidus clade were performed at 39 °C in a hybridization solution containing 50 % formamide, according to a modification (Goris *et al.*, 1998) of the microplate method described by Ezaki *et al.* (1989). Reciprocal reactions (i.e. A × B and B × A) were performed and were generally within the limits of this method (Goris *et al.*, 1998). DNA–DNA relatedness values (%) reported are based on a minimum of four hybridizations. Additional reactions, namely those between strains Vb 11.11^{T} and Vb 11.8^{T} and against the other strains in their respective clusters, were performed by the hydroxyapatite/microtitre plate method (Ziemke *et al.*, 1998) with a hybridization temperature (T_{m}) of 60 °C. The DNA G+C contents were determined using HPLC as previously described (Mesbah *et al.* 1989).

The DNA G+C content of strain Vb 11.11^{T} was 44.2 % and of strain Vb 11.8^{T} was 44.4 %, which is within the range of DNA G+C content reported for species of the genus *Vibrio*. DNA–DNA relatedness values for strains Vb 11.11^{T} (cluster 5) and Vb 11.8^{T} (cluster 70) and the type strains of the Splendidus clade tested were always below 70 % (Supplementary Table S5). DNA–DNA relatedness values between strain Vb 11.11^{T} and the other isolates of cluster 5 ranged from 78 to 94 %, and between strain Vb 11.8^{T} and the rest of the isolates of cluster 70 fluctuated between 81 and 95 %. The DNA–DNA relatedness value between strains Vb 11.11^{T} and Vb 11.8^{T} was 38 %. The DNA–DNA hybridization data demonstrated that the isolates belong to two novel species of the genus *Vibrio*.

Protein analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed in the mass unit of the University of Santiago de Compostela. Protein extraction was performed with ethanol, formic

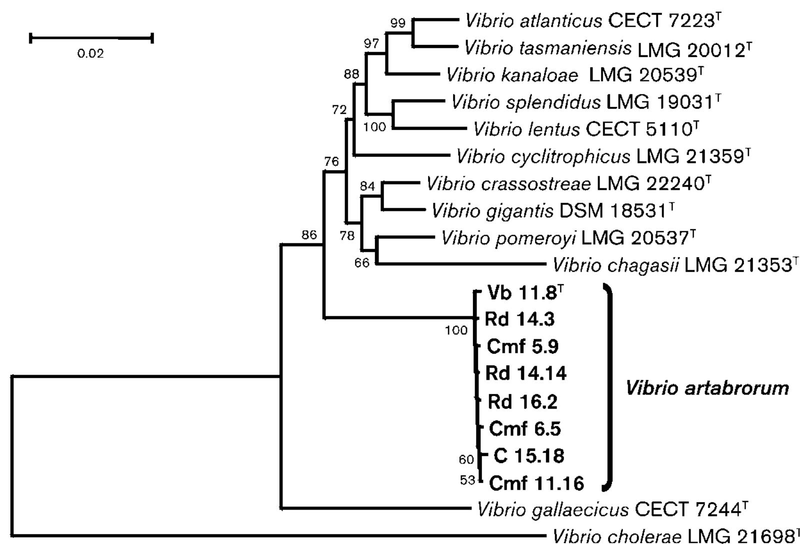

Fig. 2. Neighbour-joining tree showing the phylogenetic position of the cluster 70 strains, based on concatenated sequences of the five housekeeping genes *recA*, *pyrH*, *rpoA*, *rpoD* and *atpA*, and the 16S rRNA gene. *Vibrio cholerae* was used as the outgroup. The stability of the groupings was estimated by bootstrap percentages from 1000 replicates. Similar results were obtained with the maximum-likelihood algorithm. Bar, 0.02 substitutions per nucleotide position. Numbers at nodes show percentage bootstrap values.

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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; HCCA). Mass spectra were obtained using a MALDI-TOF Autoflex mass spectrometer (Bruker Daltonik GmbH). The measured spectra mass range was 2000–20 000 Da. Peak comparison was carried out with the database of Bruker Daltonik (http://clinprot.dbal.de). The species-limit value considered was 2300. Identification to the 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 previously obtained profiles. Strains Vb 11.11^T and Vb 11.8^T showed unique protein profiles (Supplementary Fig. S7). The closest species in both cases was *V. gigantis*, with similarity values of 2187 and 2093, respectively.

The novel isolates can be differentiated from the phylogenetically related species of the genus *Vibrio* by several phenotypic features (Table 1). Strains of both clusters can be differentiated from *V. splendidus* by their positive reaction in the Voges–Proskauer (VP) test and their ability to grow with 6 % NaCl. Strains of cluster 5 can be differentiated from *V. tasmaniensis* by their ability to produce acid from sucrose, hydrolyse Tween 80, and use D-alanine as sole carbon source, and their inability to grow at 35 °C. These strains can be differentiated from *V. kanaloae* by their positive reaction in the VP test and their negative reaction for the arginine dihydrolase test, and from *V. cyclitrophicus* by their inability to grow at 35 °C and with 8 % NaCl, as well as by their positive reaction for the VP test. Strains of cluster 70 can be differentiated from *V. kanaloae* and *V. pomeroyi* by their positive result in the VP test, and their inability to hydrolyse gelatin. In addition, the inability to hydrolyse Tween 80 or to use D-alanine as sole carbon source are also differential characteristics from *V. kanaloae*. Strains of cluster 70 can be differentiated from *V. cyclitrophicus* by their positive reaction to the VP test, and their inability to grow at 35 °C and hydrolyse gelatin and Tween 80. Supplementary Tables S6 and S7 provide the intraspecific differences of the two clusters.

In conclusion, the molecular and phenotypic data presented above show clearly that the cluster 5 and cluster 70 isolates belong to two novel species in the Splendidus clade. The names *Vibrio atlanticus* sp. nov. and *Vibrio artabrorum* sp. nov. are proposed for these novel species.

Description of *Vibrio atlanticus* sp. nov.

Vibrio atlanticus (at.lan′ti.cus. L. masc. adj. *atlanticus* Atlantic, from the Atlantic Ocean).

Gram-negative, facultatively anaerobic, motile, flagellated rods (0.7–0.8 × 1.1–1.4 μm in size). Colonies are beige in colour, round and smooth on MA. Swarming is not observed. Colonies are 2–6 mm in diameter on MA plates after 24 h of incubation at 25 °C. Not luminescent. Oxidase- and catalase-positive. Negative for arginine dihydrolase, and lysine and ornithine decarboxylases. Reduces nitrate to nitrite. Voges–Proskauer test is positive

Table 1. Phenotypic characteristics for distinguishing *V. atlanticus* sp. nov. and *V. artabrorum* sp. nov. from phenotypically and phylogenetically related species of the genus *Vibrio*

Taxa: 1, *V. atlanticus* sp. nov. (five strains); 2, *V. artabrorum* sp. nov. (eight strains); 3, *V. celticus* CECT 7224^T; 4. *V. gigantis* DSM 18531^T; 5, *V. splendidus* CECT 528^T; 6, *V. crassostreae* LMG 22240^T; 7, *V. pomeroyi* LMG 20537^T; 8, *V. tasmaniensis* LMG 20012^T; 9, *V. lentus* CECT 5110^T; 10, *V. kanaloae* LMG 20539^T; 11, *V. chagasii* LMG 21353^T; 12, *V. cyclitrophicus* LMG 21359^T; 13, *V. gallaecicus* LMG 24045^T. Differential tests were performed in the laboratory on all *V. atlanticus* sp. nov. and *V. artabrorum* sp. nov. isolates as well as the type strains of the other vibrios within the Splendidus clade, using classical tube and plate methods and API 20E strips. +, Positive; −, negative; V, variable; V+, variable but type strain is positive; V−, variable but type strain is negative. The number of strains giving a positive result is indicated in parentheses.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Arginine dihydrolase	−	V− (2)	+	+	−	+	+	−	−	+	+	−	−
ONPG	V+ (3)	V+ (4)	−	−	+	−	+	−	+	−	−	+	−
Voges–Proskauer	+	+	+	−	−	−	−	+	+	−	−	−	−
Acid from:													
Sucrose	+	+	−	−	+	+	+	−	−	+	−	+	−
L-Arabinose	V+ (1)	V+ (4)	−	−	−	−	+	−	−	−	−	−	−
Susceptibility to O/129 (150 μg)	V+ (4)	V+ (7)	+	+	+	+	+	+	−	+	+	+	+
Growth at/with:													
35 °C	−	V− (1)	−	−	−	−	−	+	−	−	−	+	−
6 % NaCl	+	+	+	−	−	+	+	+	+	+	+	+	−
8 % NaCl	−	V− (1)	−	−	−	−	+	−	−	−	+	+	−
Hydrolysis of:													
Gelatin	V− (4)	−	+	+	+	+	+	−	+	+	+	+	+
Tween 80	+	−	+	+	+	+	−	−	+	+	+	+	+
Use of D-alanine as sole carbon source	+	−	+	ND	+	ND	+	−	ND	+	+	ND	ND

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and indole test is usually positive. All strains hydrolyse starch and Tween 80. Gelatin is hydrolysed by all isolates except the type strain, Vb 11.11^T. Growth occurs at 4 °C and with 3–6 % NaCl (w/v), but not at 3 or 44 °C and salinities greater than 8 % NaCl. Most strains are susceptible to the vibriostatic agent O/129 (150 µg per disc). Acid is produced from glycerol, D-ribose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, aesculin, cellobiose, maltose, trehalose, starch, glycogen and potassium gluconate. None of the strains produce acid from erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, melibiose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate. In addition, the type strain, Vb 11.11^T, produced acid from L-arabinose, D-xylose, sucrose, D-galactose, D-mannitol, gentiobiose and L-fucose. All strains can use as sole carbon source D-ribose, D-fructose, trehalose, maltose, cellobiose, sucrose, gluconic acid, D-mannitol, glycerol, succinic acid, L-threonine, L-glutamic acid, D-alanine, L-arginine and L-tyrosine, but not D-xylose, L-rhamnose, lactose, D-salicin, D-amygdalin, D-sorbitol, *myo*-inositol, butyric acid, L-ornithine, amino-*N*-butyric acid, L-lysine, putrescine, 3-hydroxybutyric acid or L-fucose. In addition, the type strain, Vb 11.11^T, can use as sole carbon source D-glucose, sodium acetate, propionic acid, citric acid, L-serine, L-citrulline, aspartic acid and L-histidine.

The type strain, Vb 11.11^T (=CECT 7223^T =LMG 24300^T), was isolated from clam, *Ruditapes philippinarum*, in Galicia, north-west Spain, in January 2005. The DNA G+C content of strain Vb 11.11^T is 44.2 mol%.

Description of *Vibrio artabrorum* sp. nov.

Vibrio artabrorum [ar.ta.bro′rum. L. gen. pl. n. *artabrorum* intended to mean that this species was isolated from 'Portus Magnus Artabrorum' (the largest harbour of the Artabri), the Latin name of the main gulf in the Galician coasts].

Gram-negative, facultatively anaerobic rods (0.8–1.0 × 1.4–1.8 µm in size). On MA plates after 24 h of incubation at 25 °C, non-swarming, beige, round and smooth 2–6 mm diameter colonies are observed. Strain Cmf 6.5 produces a brownish diffusible pigment. Not luminescent. All isolates form yellow colonies on TCBS agar. Growth occurs with 3–6 % NaCl (w/v) (one isolate is able to grow in media with 0.5–8 % NaCl). Growth occurs at 4 °C, but not at 35 °C (one exception). Mostly susceptible to the vibriostatic agent O/129 (150 µg per disc). All isolates are positive for oxidase and catalase and reduce nitrates to nitrites. Voges–Proskauer test is positive, but lysine and ornithine decarboxylase, indole, gelatinase and lipase tests are negative. All strains produce acid from D-ribose, D-galactose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, cellobiose, maltose, sucrose, trehalose, starch, glycogen and potassium gluconate. None of the strains produce acid from erythritol, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, inositol, D-sorbitol, methyl α-D-mannopyranoside, amygdalin, salicin, inulin, melezitose, raffinose, xylitol, D-fucose, L-arabitol or potassium 5-ketogluconate. The type strain, Vb 11.8^T, also produced acid from glycerol, L-arabinose, D-xylose and D-rhamnose. All isolates can use as sole carbon source D-ribose, D-fructose, trehalose, D-mannose, maltose, cellobiose, sucrose, gluconic acid, glucuronic acid, D-mannitol, glycerol, succinic acid, L-arginine, L-citrulline and aspartic acid, but not L-rhamnose, salicin, amygdalin, D-sorbitol, *myo*-inositol, butyric acid, L-ornithine, amino-*N*-butyric acid, L-lysine, putrescine or 3-hydroxybutyric acid. The type strain, Vb 11.8^T, can also use as sole carbon source D-xylose, D-glucose, D-galactose, galacturonic acid, sodium acetate, threonine and α-ketoglutaric acid.

The type strain, Vb 11.8^T (=CECT 7226^T =LMG 23865^T), was isolated from clam, *Ruditapes philippinarum*, in Galicia, north-west Spain, in January 2005. The DNA G+C content of strain Vb 11.8^T is 44.4 mol%.

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