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Vibrio breoganii sp. nov., a non-motile, alginolytic, marine bacterium within the *Vibrio halioticoli* clade

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Seven non-motile, facultatively anaerobic, alginolytic marine bacteria were isolated from the cultured clams *Ruditapes philippinarum* and *Ruditapes decussatus*. Phylogenetic analysis based on 16S rRNA gene sequences showed that these marine bacteria were closely related to the recently described species *Vibrio comitans*, *Vibrio rarus* and *Vibrio inusitatus* (⩾99.0 % sequence similarity). Phylogenetic analysis based on the housekeeping genes *rpoA*, *recA* and *atpA* grouped the isolates together and allocated them to the *Vibrio halioticoli* species group. Amplified fragment length polymorphism DNA fingerprinting also grouped them together and enabled them to be differentiated from recognized species of the *V. halioticoli* clade. DNA–DNA hybridizations showed that the isolates belonged to a novel species; phenotypic features such as the ability to grow at 4 °C and in the presence of 6 % NaCl also enabled them to be separated from other species. The DNA G+C content of RD 15.11^T is 44.4 mol%. The genotypic and phenotypic data showed that the isolates represent a novel species in the *V. halioticoli* clade. The name *Vibrio breoganii* sp. nov. is proposed, with RD 15.11^T (=CECT 7222^T =LMG 23858^T) as the type strain.

During the last decade, the number of *Vibrio* species has increased rapidly. At present, there are more than 70 recognized *Vibrio* species. It is known that these fermentative bacteria constitute a high proportion of the heterotrophic bacteria associated with marine bivalves, especially oysters, mussels and clams (Kueh & Chan, 1985; Pujalte *et al.*, 1993; Gómez-León *et al.*, 2005). Several *Vibrio* species can be pathogenic and are associated with disease outbreaks (Prado *et al.*, 2005); therefore, accurate identification of these micro-organisms is important. The multilocus sequence analysis technique has greatly improved the taxonomy of strains belonging to the *Vibrionaceae* and many existing species have been assigned to specific groups. These groups are usually named after a representative species in the group, e.g. the *splendidus*, *diazotrophicus*, *scophthalmi*, *fischeri*, *harveyi* or *halioticoli* clades, among others (Thompson *et al.*, 2007; Sawabe *et al.*, 2007b). Species of the *Vibrio halioticoli* clade are found in association with ecological niches in the gut of *Haliotis* species and are alginolytic, non-motile, unflagellated and fermentative marine bacteria. The *V. halioticoli* clade consists at present of eight species: *V. halioticoli*, *V. superstes*, *V. neonatus*, *V. ezurae*, *V. gallicus*, *V. comitans*, *V. inusitatus* and *V. rarus*. *V. halioticoli* was first described by Sawabe *et al.* (1998). *V. comitans*, *V. inusitatus* and *V. rarus* were described more recently (Sawabe *et al.*, 2007a). Strains of the *V. halioticoli* clade have been found all over the world, including Japan (Sawabe *et al.*, 1995, 1998, 2002, 2004a, 2007a), Australia (Hayashi *et al.*, 2003), South Africa (Sawabe *et al.*, 2003) and France (Sawabe *et al.*, 2004b). In this study, a group of seven isolates that were phylogenetically and phenotypically similar to species of the *V. halioticoli* clade was isolated from the microbiota of Spanish clams (*Ruditapes philippinarum* and *Ruditapes decussatus*) and shown to represent a novel species. This is the first time that isolates of this clade have been obtained from molluscs other than *Haliotis* species.

Seven isolates [RD 15.11^T (=CECT 7222^T =LMG 23858^T), RD 2G5 (=CECT 7367 =LMG 24484), VB 16.3, C5.5, RD 2B2, C 4.15 and CMJ 13.7] were obtained from cultured clams on the north-west coast of Spain (Galicia) during a 2 year survey from March 2004 to December 2005. Standard bacteriological procedures were employed for

Abbreviation: AFLP, amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers determined in this study are available with the online version of this paper.

Differential phenotypic characteristics of the seven isolates of *V. breoganii* sp. nov. and phylogenetic trees based on concatenated *rpoA*, *atpA* and 16S rRNA gene sequences are available with the online version of this paper.

analysis of the clams (Prado *et al.*, 2005). Briefly, groups of 15–20 clams, depending on size, were dissected aseptically and samples of hepatopancreas, mantle, gonad, gills and extrapalial fluid were collected. Homogenates were prepared by adding a volume of saline solution (SS, 0.85 %) to the weighed organ samples (1 : 1). Samples were diluted serially in SS, plated on marine agar (MA; Pronadisa) and thiosulphate–citrate–bile–sucrose agar (TCBS; Oxoid) and incubated at 23±1 °C for 10 days (MA) or 48 h (TCBS). Pure cultures of the different colony morphologies were recovered on MA.

The reference strains used, *V. comitans* LMG 23416^T, *V. rarus* LMG 23674^T, *V. inusitatus* LMG 23434^T, *V. ezurae* LMG 19970^T, *V. neonatus* LMG 19973^T, *V. gallicus* LMG 21330^T and *V. superstes* LMG 21323^T, were obtained from the BCCM/LMG. All strains were cultured on MA at 25 °C for 24 h. All cultures were maintained frozen at −80 °C in marine broth (Pronadisa) supplemented with 15 % (v/v) glycerol.

Phenotypic characteristics were determined by standard methods and by using commercial miniaturized kits (API 50CH, API 20E and API ZYM; bioMérieux). API 50CH was used with slight modifications as reported previously (Prado, 2006). Briefly, bacterial suspensions were prepared in SS, adjusted to an OD_{580} of 1.0 and mixed with ZOF medium (Lemos *et al.*, 1985) (1 : 10, v/v) prior to inoculation of the galleries. Readings were taken at 24, 48, 96 and 120 h and after 6 days. Readings after 6 days incubation were used for comparison. For API 20E and API ZYM, standard methodologies were used except that the medium used for the bacterial suspension was SS. In all cases, incubation was done at 23±1 °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. The absence of any flagellar arrangement was determined after staining cells from cultures grown on MA by the method of Heimbrook *et al.* (1989).

The seven clam isolates examined in this study shared the main phenotypic and chemotaxonomic features of the genus *Vibrio* except for the absence of flagella (Farmer, 1992). The strains were facultatively anaerobic, non-motile, Gram-negative and oxidase-positive. They required salt for growth, grew on TCBS, were susceptible to the vibriostatic agent O/129 (150 μg per disc) and reduced nitrates to nitrites. The seven isolates were phenotypically homogeneous in the tests performed except for 11 variable traits (see Supplementary Table S1, available in IJSEM Online).

Genomic DNA for sequencing was extracted as described previously (Prado *et al.*, 2005). Amplification and sequencing of the 16S rRNA gene was performed by using a GenomeLab DTCS-Quick Start kit (Beckman Coulter) (Prado *et al.*, 2005). Amplification and sequencing of the housekeeping genes *rpoA*, *recA* and *atpA* were performed according to Thompson *et al.* (2004, 2005, 2007). For the reference strains, sequences were retrieved from GenBank/EMBL; sequences that were unavailable were determined in this study. 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 constructed by using the neighbour-joining and maximum-parsimony algorithms (Tamura *et al.*, 2007). Distance matrices were calculated by using Kimura's two-parameter correction and stability of the groupings was estimated by bootstrap analysis (1000 replicates) using the program MEGA version 4.0.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that the seven isolates belonged to the *Gammaproteobacteria*. The isolates showed >99 % 16S rRNA gene sequence similarity to each other and were related phylogenetically to species of the *V. halioticoli* clade (Fig. 1; Supplementary Figs S1 and S2, available in IJSEM Online). RD 15.11^T showed >98 % 16S rRNA gene sequence similarity to the type strains of *V. comitans* (99.6 %), *V. inusitatus* (99.0 %), *V. rarus* (99.0 %), *V. ezurae* (98.8 %), *V. halioticoli* (98.6 %), *V. superstes* (98.7 %) and *V. neonatus* (97.7 %). Recently, sequencing of housekeeping genes has proven useful to determine phylogenetic relationships among micro-organisms. For the family *Vibrionaceae*, different loci, e.g. *gapA*, *gyrB*, *recA*, *rpoA*, *pyrH*, *atpA* and *dnaJ*, have been studied in the search for a useful phylogenetic marker capable of delineating *Vibrio* species (Thompson *et al.*, 2004, 2005, 2007; Sawabe *et al.*, 2007b; Nhung *et al.*, 2007). In the present study, fragments of the *rpoA* (931 bp), *recA* (613–713 bp) and *atpA* (1322 bp) genes were sequenced. According to Thompson *et al.* (2005), strains of the same species will have ⩾98.0 % *rpoA* and ⩾94.0 % *recA* gene sequence similarity. The similarities between the *rpoA* gene sequence of *V. breoganii* RD 15.11^T and the type strains of *V. comitans* and *V. superstes* were above the intraspecies limit; similarities with the other six related species were under the limit. For the *recA* gene sequences, all similarity values were under the intraspecies limit (Table 1). It was impossible to amplify the *recA* gene for the type strain of *V. superstes* and this sequence is not available in GenBank. Phylogenetic trees based on *rpoA*, *recA* and *atpA* gene sequences confirmed the allocation of the isolates to the *V. halioticoli* clade, enabled their differentiation from recognized species of this clade and revealed *V. superstes* and *V. comitans* as the nearest phylogenetic neighbours (Fig. 2).

Genomic DNA for amplified fragment length polymorphism (AFLP) analysis was extracted by using an Easy DNA kit (Invitrogen). AFLP analysis was performed as described previously (Thompson *et al.*, 2001), except that dendrograms were constructed by using the DICE coefficients and the UPGMA algorithm. AFLP DNA fingerprinting grouped the isolates together and enabled them to be differentiated from recognized species of the *V. halioticoli* clade, as well as from other vibrios (Beaz Hidalgo *et al.*, 2008).

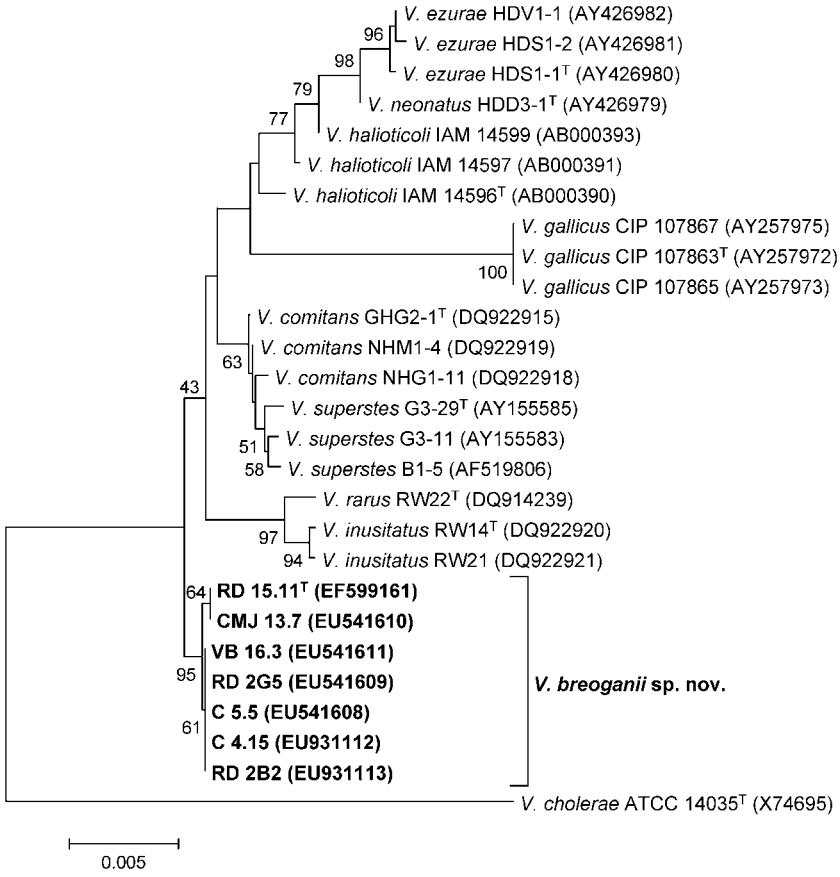

Fig. 1. Phylogenetic tree based on partial 16S rRNA gene sequences using the neighbour-joining algorithm. *Vibrio cholerae* was used as outgroup. The stability of the groupings was estimated by bootstrap percentages from 1000 replicates. Similar results were obtained with the maximum-parsimony algorithm. Bar, 0.005 substitutions per nucleotide position.

Genomic DNA for DNA–DNA hybridizations and determination of the DNA G+C content was prepared according to a modification (Cleenwerck *et al.*, 2002) of the procedure of Wilson (1987). DNA–DNA hybridizations were performed at 39 °C according to a modification (Goris *et al.*, 1998) of the method described by Ezaki *et al.* (1989). Reciprocal reactions (e.g. A × B and B × A) were performed and were within the limits of this method (Goris *et al.*, 1998). The DNA G+C content of RD 15.11^T was determined by using the method of Mesbah *et al.* (1989). DNA of strain RD 15.11^T was hybridized with DNA of the nearest phylogenetic neighbours; DNA–DNA relatedness values of RD 15.11^T were 58 % with *V. superstes* LMG 21323^T, 36 % with *V. comitans* LMG 23416^T, 28 % with *V. inusitatus* LMG 23434^T, 19 % with *V. ezurae* LMG 19970^T, 18 % with *V. neonatus* LMG 19973^T, 15 % with *V. rarus* LMG 23674^T and 14 % with *V. halioticoli* LMG 18542^T.

Table 1. Sequence similarity values (%) between the 16S rRNA, *recA*, *rpoA* and *atpA* gene sequences of *V. breoganii* RD 15.11^T and type strains of related *Vibrio* species

Type strain	16S rRNA	*recA*	*rpoA*	*atpA*
V. comitans LMG 23416^T	99.4	93.0	99.8	97.4
V. inusitatus LMG 23434^T	99.0	91.0	96.0	97.2
V. rarus LMG 23674^T	99.1	84.0	94.0	93.0
V. superstes DSM 16383^T	98.2	ND	99.8	98.4
V. halioticoli IAM 14596^T	98.3	86.0	95.5	93.8
V. ezurae DSM 17533^T	97.6	86.6	95.7	94.4
V. neonatus DSM 17531^T	97.0	86.1	95.5	93.8
V. gallicus DSM 16639^T	97.5	82.0	92.0	88.5

ND, Not done.

The novel isolates can be differentiated from phylogenetically related species by several features (Table 2). Strains can be differentiated from *V. superstes* by their ability to grow at 4 °C and in 6 % NaCl, their positive reaction for the ONPG test and their inability to produce acid from trehalose. With the API ZYM methodology, other differences such as butyrate esterase (C4) and naphthol-AS-BI-phosphohydrolase were found (data not shown). The novel isolates can be differentiated from *V. inusitatus* by their ability to produce acid from D-galactose and their positive reaction in the ONPG test. They can be differentiated from *V. rarus* by their ability to grow at 4 °C, their positive reaction in the ONPG test, their ability to produce acid from amygdalin, D-xylose, D-galactose, aesculin and salicin, their inability to produce acid from glycerol and their negative reaction in the indole test. Additionally, the isolates showed a positive reaction in the API ZYM tests for esterase (C4), esterase lipase (C8), acid phosphatase and

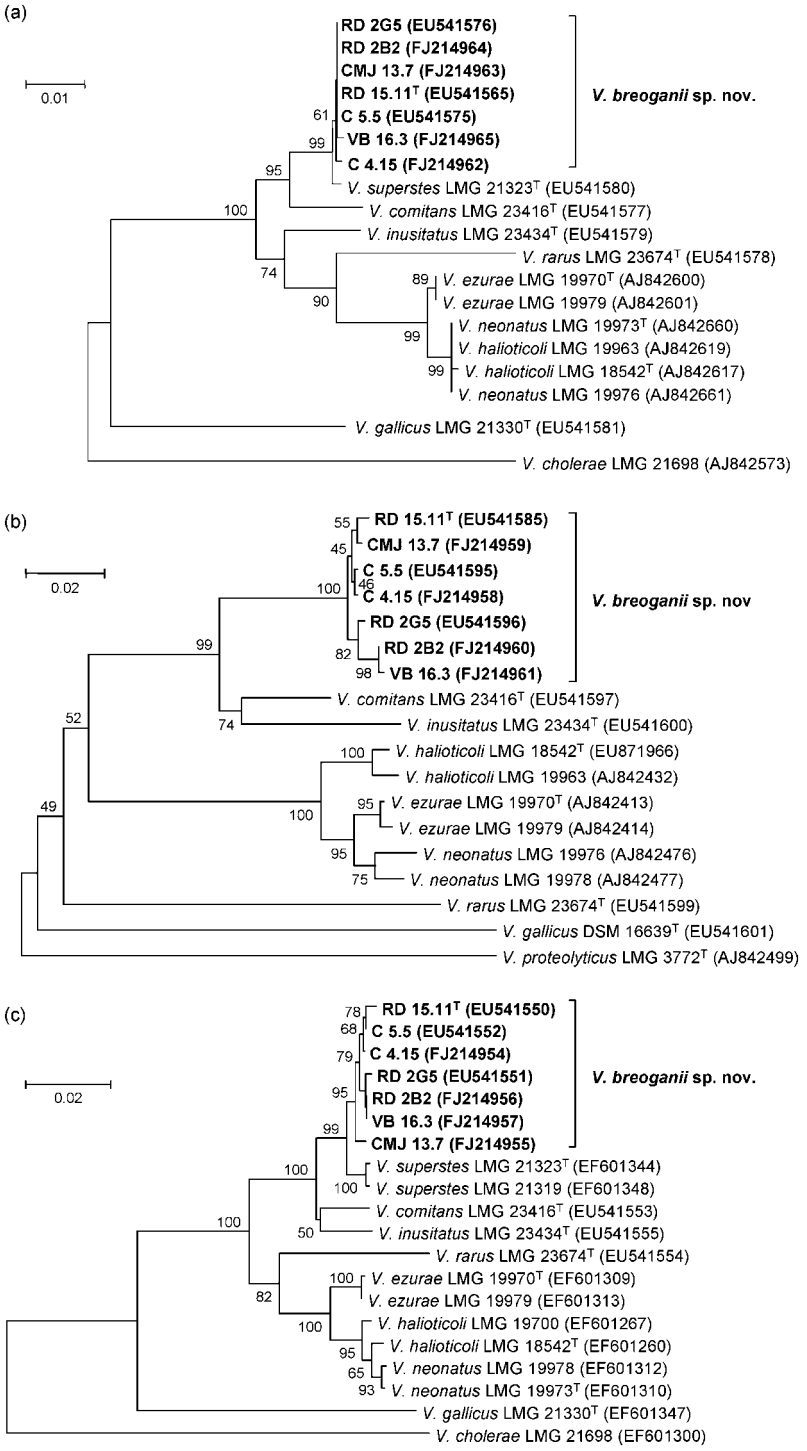

Fig. 2. Phylogenetic trees based on partial *rpoA* (a), *recA* (b) and *atpA* (c) sequences obtained by using the neighbour-joining algorithm. *Vibrio cholerae* or *Vibrio proteolyticus* was used as outgroup. The stability of the groupings was estimated by bootstrap percentages from 1000 replicates. Similar results were obtained employing the maximum-parsimony algorithm. Bars, no. of substitutions per nucleotide position.

naphthol-AS-BI-phosphohydrolase, in contrast to *V. rarus*, *V. inusitatus* and *V. comitans*.

In conclusion, the data presented above show clearly that the seven isolates belong to a novel species in the *V. halioticoli* clade. The name *Vibrio breoganii* sp. nov. is proposed for this novel species. The type strain is RD 15.11^{T} (=LMG 23858^{T}=CECT 7222^{T}).

Description of *Vibrio breoganii* sp. nov.

Vibrio breoganii [bre.o.ga′ni.i. N.L. masc. gen. n. *breoganii* of Breogan, a mythical Celtic king of Galicia (north-west Spain), from where the strains were isolated].

Gram-negative, facultatively anaerobic, non-motile, non-flagellated rods (0.5–0.75 × 1.0–2.0 μm). Forms non-pigmented, translucent, non-swarming, round, smooth col-

Table 2. Distinguishing phenotypic characteristics of *V. breoganii* and phenotypically and phylogenetically related *Vibrio* species

Species: 1, *V. breoganii* sp. nov. (*n*=7); 2, *V. comitans*; 3, *V. inusitatus*; 4, *V. rarus*; 5, *V. ezurae*; 6, *V. neonatus*; 7, *V. halioticoli*; 8, *V. gallicus*; 9, *V. superstes*. Except for *V. breoganii*, data are from type strains and were obtained in our laboratory. +, Positive; −, negative; V+, variable, but type strain is positive (numbers of strains testing positive are in parentheses); V−, variable, but type strain is negative (numbers of strains testing positive are in parentheses). All species are fermentative, sensitive to the vibriostatic agent O/129 (150 μg), non-motile, oxidase- and catalase-positive, require Na^+ for growth, reduce nitrate, grow in 3 % NaCl and at 15–30 °C, grow on TCBS, produce alginase, and produce acid from D-glucose and D-mannitol. All species are negative for pigmentation, swarming, growth above 37 °C and in <0.5 % NaCl, gas production from D-glucose, arginine dihydrolase, lysine and ornithine decarboxylases, luminescence, hydrolysis of agar, production of amylase, gelatinase and lipase, and acid production from D-sorbitol, L-arabinose and inositol.

Characteristic	1	2	3	4	5	6	7	8	9
Indole test	−	−	−	+	+	+	+	+	−
ONPG test	+	+	−	−	−	−	+	−	−
Acid production from:									
Amygdalin	+	+	+	−	+	−	−	−	+
Glycerol	−	−	−	+	−	−	+	−	−
D-Ribose	V− (2)	−	−	−	−	−	+	+	+
D-Xylose	+	+	+	−	−	−	−	−	+
D-Galactose	+	+	−	−	−	+	−	−	+
Aesculin	+	+	+	−	−	−	−	−	+
Salicin	+	+	+	−	−	−	−	−	+
Cellobiose	+	+	+	+	+	−	−	−	+
Trehalose	−	−	−	−	−	−	−	−	+
Gentiobiose	+	+	+	+	+	−	−	−	+
Growth at/in:									
4 °C	+	+	+	−	−	−	−	−	−
6 % NaCl	+	+	+	+	−	−	−	−	−
DNase	−	+	−	−	−	−	−	−	−
API ZYM tests:									
Esterase (C4)	+	−	−	−	−	−	−	+	−
Esterase lipase (C8)	V+ (6)	−	−	−	−	+	−	+	−
Acid phosphatase	+	−	−	−	−	−	−	−	+
Naphthol-AS-BI-phosphohydrolase	+	−	−	−	−	−	−	−	−

onies that are 0.3–0.7 mm in diameter on MA plates after 24 h incubation at 25 °C. Not luminescent. All strains apart from strain RD 2G5, which forms colonies with a translucent yellow appearance, form opaque, round (0.7–1.0 mm diameter), green colonies on TCBS agar. No growth occurs without NaCl in the culture medium. Mesophilic and chemo-organotrophic. Growth occurs in 1.5–6.0 % NaCl (w/v) and at 4 °C, but not at salinities above 8.0 % NaCl or above 37 °C. Susceptible to the vibriostatic agent O/129 (150 μg per disc); all strains apart from C 5.5 are resistant to ampicillin (10 μg per disc). Negative for arginine dihydrolase, and lysine and ornithine decarboxylases. Ferments glucose without producing gas and positive for oxidase and catalase. Reduces nitrates to nitrites. Indole, urease, Voges–Proskauer, H_2S, gelatinase, amylase, use of citrate, lipase and DNase tests are all negative. Positive for methyl red, ONPG and alginase activity tests. API ZYM reaction tests are positive for alkaline phosphatase, butyrate esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. Produces acid from D-xylose, D-galactose, D-fructose, D-mannitol, *N*-acetylglucosamine, amygdalin, aesculin, salicin, cellobiose, maltose, gentiobiose, potassium gluconate and potassium 2-ketogluconate. Does not produce acid from glycerol, erythritol, D-arabinose, L-arabinose, D-adonitol, methyl-α-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, trehalose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol or potassium 5-ketogluconate.

The type strain RD 15.11^T (=CECT 7222^T =LMG 23858^T) and six reference strains [RD 2G5 (=CECT 7367 =LMG 24484), VB 16.3, C5.5, RD 2B2, C 4.15 and CMJ 13.7] were isolated from the clams *Ruditapes decussatus* and *Ruditapes philippinarum* in Galicia, north-west Spain. The DNA G+C content of strain RD 15.11^T is 44.4 mol%. Strain RD 15.11^T produces acid from melibiose, L-xylose, arbutin, glycogen and D-lactose, but not from sucrose, D-ribose, D-mannose or L-rhamnose.

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